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Oxidative State of Songbirds During Migration and Best Practices to Communicate the Science

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OXIDATIVE STATE OF SONGBIRDS DURING MIGRATION AND BEST PRACTICES TO COMMUNICATE THE SCIENCE

BY

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ABSTRACT

Managing oxidative stress is an important physiological function for all aerobic organisms, particularly during periods of prolonged high metabolic activity, such as long-distance migration. However, no previous study has investigated the oxidative status of songbirds at different stages of migration, whether that oxidative status depends on the condition of the birds, how birds use their condition in concert with access to free water to make stopover decisions, or the effects of oxidative challenges like flight on subsequent reproduction. Additionally, scientists undertaking such research are commonly expected to disseminate their results as a Broader Impact of their studies—typically to secure research funding—and often struggle to do so, given that they are not usually trained in non-technical communication or public engagement. In this dissertation, I introduce the fundamentals of oxidative balance in flying songbirds (Chapter 1), discuss physiological factors affecting the condition and behavior of migratory songbirds on stopover (Chapters 2 and 3), describe the impact of dietary antioxidants and flight exercise on songbird reproduction (Chapter 4), and present a framework of guiding principles to help researchers communicate their science (Chapter 5). Development of this framework influenced the style and approach of the dissertation as a whole, with each chapter's organization and figure design benefiting from lessons transferrable from communication with non-scientist audiences to communication with professional peers.

To investigate the relationship between energy stores and oxidative status of long-distance migratory birds on stopover, I drew blood samples from two species of Neotropical migrant (Blackpoll Warbler, *Setophaga striata*, and Red-eyed Vireo,

Vireo olivaceus) with differing migration strategies during autumn migration on Block Island, USA, and a species of trans-Saharan migrant (Garden Warbler, *Sylvia borin*) during spring migration on the island of Ponza, Italy; I found fat stores to be positively correlated with circulating antioxidant capacity in Blackpoll Warblers and Red-eyed Vireos and positively correlated with circulating lipid oxidation levels in all three species. Among Garden Warblers, oxidative damage levels decreased with time on stopover (up to 8 nights). Thus, the physiological strategy of migrating songbirds appears to be to (1) build prophylactic antioxidant capacity in concert with fuel stores at stopover sites before a long-distance flight, and (2) repair oxidative damage while refueling at stopover sites after long-distance flight.

To assess the contribution of free water to migratory birds' stopover decisions, I captured 61 free-living Garden Warblers in spring at a frequently used stopover site in the Mediterranean Sea, housed them with or without drinking water, and measured nocturnal restlessness (Zugunruhe) in relation to energy stores at capture; water-deprived birds of high fat score showed the highest Zugunruhe activity, suggesting that individuals with higher fat scores might be expected, regardless of flight muscle size, to depart a dry stopover site more readily than a site with freely available water.

To examine how songbirds use nutrition to manage trade-offs in antioxidant allocation between endurance flight and subsequent reproduction, I performed a controlled experiment with Zebra Finches (*Taeniopygia guttata*), using four treatment groups of birds that either were or were not provided a water- and lipid-soluble antioxidant-supplemented diet and were or were not trained to perform daily endurance flight for 6 weeks before breeding; I found that exercised birds had higher

oxidative damage levels than non-exercised birds after flight training, supplementation with water-soluble antioxidants decreased the deposition of lipid-soluble antioxidants to eggs and yolk size, and flight exercise lowered deposition of lutein but not vitamin E to eggs. These results mechanistically demonstrate potential carry-over effects of nutrition and exertion on the capacity of songbirds to provision eggs with important nutrients after long-distance flights.

To address how the potential for "impact" of varied Broader Impacts dissemination activities can be critically assessed during proposal writing and peer review, I combined the experiences of successful practitioners with communication theory to synthesize a five-point framework; this "Broader Impacts Impact Framework" summarizes best practices in communication and outreach, can be easily used by scientists during proposal writing and review, and focuses on five main factors: who, why, what, how, and with whom.

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PREFACE

The following dissertation appears in Manuscript Format, with chapters prepared in accordance with the formatting guidelines of their respective target publications. Four of the five chapters have been published in peer-reviewed journals, and one has been accepted for publication with minor revision. Chapter 1 is an invited review paper published by *Journal of Field Ornithology* in January 2016 (volume 87, issue 1, pages 1-20; doi: 10.1111/jofo.12135); this paper summarizes for field ornithologists state-of-the-science knowledge regarding oxidative balance in migrating songbirds, from a physiological and nutritional perspective. Chapter 2 is a field investigation of the oxidative status of migratory songbirds on stopover, published by *Ecology and Evolution* in July 2015 (volume 5, issue 15, pages 3198-3209; doi: 10.1002/ece3.1601); this paper describes the oxidative state of two species of songbirds preparing for autumn migration on Block Island, USA, and one species of songbird recovering from a long-distance flight during spring migration on the island of Ponza, Italy. Chapter 3 reports a water-deprivation experiment conducted on the island of Ponza with a migratory songbird, published by *Journal of Ornithology* in March 2015 (volume 156, supplement 1, pages 425-432; doi: 10.1007/s10336-015-1198-1); this article demonstrates that restricted water access may increase the nocturnal restlessness, and hence propensity to leave a stopover site, of individual songbirds with large energy stores. Chapter 4 has been accepted for publication at *Journal of Experimental Biology* and reports an experiment with a model songbird species (Zebra Finch) investigating carry-over effects of diet and exercise on subsequent reproduction, relevant to migratory birds that breed soon after spring

migration; this paper shows for the first time that access to water-soluble dietary antioxidants and flight exercise both influence how female birds allocate lipid-soluble antioxidants and other nutrients to their eggs. Chapter 5 synthesizes scholarship and best practices in science communication to build a framework that can clearly and carefully help define truly "broad" and "impactful" Broader Impacts activities, published in *Frontiers in Ecology and the Environment* in June 2015 (volume 13, issue 5, pages 273-279; doi: 10.1890/140209); this article addresses the need among ecologists, and other scientists, for a standardizing framework that can aid the crafting and peer review of Broader Impacts outreach activities.

The unifying theme of this dissertation is the physiology of migratory songbirds and effective communication of research results; the body of work encompasses two experiments with captive songbirds, a pair of field investigations with wild songbirds, a topical review, and a guiding framework to facilitate communication of science outside of the peer-reviewed literature. The process of developing this framework also improved my communication within the peer-reviewed literature, enhancing message organization and design of visuals in the physiological articles to ease their comprehension.

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Oxidative balance in birds: an atoms-to-organisms-to-ecology primer for ornithologists

by

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ABSTRACT

All air-breathing organisms must face the challenge of oxidative damage, and understanding how animals cope can lend insight into their ecology. Unlike other vertebrates, birds rely primarily on fats to fuel endurance exercise such as migration, and therefore face a greater potential for damage from the reactive by-products of their own metabolism. We review the physiological ecology of migrating birds through the lens of oxidation-reduction chemistry, underscoring how oxidative balance in wild birds may affect their dietary choices and use of critical stopover habitats during migration. Recent studies reveal that migratory birds prepare for oxidative challenges either by up-regulating endogenous antioxidants or by consuming them in their diet, and they repair oxidative damage after long flights, although much remains to be discovered about how birds maintain oxidative balance over the course of migration. We conclude by describing some of the most used and useful measures of antioxidant status and oxidative damage that field ornithologists can include in their tool kit of techniques to probe the oxidative balance of wild birds.

Key words: antioxidants, lipid oxidation, measurement, migration, stopover

Compared to other vertebrates, birds are unique endurance athletes capable of sustaining high metabolic rates while relying on fat as their primary fuel. All vertebrates generate reactive by-products during oxygen-based metabolism, and exercise requires an increase in energy production and consequently can increase oxidative by-products. Endurance flights of migrating birds are particularly remarkable because the high metabolic rates required to stay aloft for long periods are fueled by fats that produce more oxidative by-products per capita than other fuels such as proteins and carbohydrates. In this review, we present evidence from recent studies that reveal how migratory birds overcome or accommodate the risks of oxidative stress during long-duration flights, and discuss how this understanding can provide deep insight into the physiological and behavioral ecology of birds during migration.

Oxidative stress and its association with aging, disease, and life-history trade-offs have received considerable recent attention (e.g., Cohen et al. 2008, 2010, Costantini 2008, 2014, Monaghan et al. 2009, Buttemer et al. 2010, Costantini et al. 2010, Lushchak 2011, McGraw 2011, Pamplona and Costantini 2011, Beaulieu and Costantini 2014). In this review, we focus on the nutritional and physiological ecology of birds, with special reference to the oxidative challenges of migration, to highlight the relevance of oxidative balance research to field investigations. We first summarize current understanding of the atomic origin of oxidative damage and the particular vulnerability of fats used by birds, followed by an overview of the various components of the antioxidant system that help maintain oxidative balance at the organismal level. We then place these aspects of biochemistry within an ecological context, describing the oxidative costs of long-distance flight and how the dietary choices of migrating

birds on stopover may help prevent oxidative stress. We conclude with a primer of measurements practical for field researchers to study the oxidative status of migratory birds, and end with a brief discussion of gaps in our knowledge that may be addressed by future field research into oxidative balance.

ATOMIC LEVEL: THE ESSENTIALS OF OXIDATIVE BALANCE

Any investigation into oxidative balance requires at least a basic understanding of oxidation-reduction chemistry. Amateur and professional ornithologists alike will recall high school chemistry lectures in which the handy mnemonic device OIL RIG was presented: oxidation is loss, and reduction is gain, of electrons. Oxidation-reduction chemistry explains both the source of oxidative damage in bird tissues and how birds can counter damage-causing molecules. This understanding allows field ornithologists to more wisely choose among the possible measurements available to probe the oxidative status of migratory birds.

How does oxidative damage happen? Oxidative balance in birds begins at the molecular level (Fig. 1.1) and with the chemical reactions taking place in mitochondria, the organelles responsible for generating adenosine triphosphate (ATP), the energetic currency of cells. Inside each mitochondrion—on the inner mitochondrial membrane—enzyme complexes collectively known as the electron transport chain (ETC) transfer electrons generated from the metabolism of food to a final electron acceptor, molecular oxygen (O_2), reducing it to water (H_2O) in the process. The energy released during this transfer of electrons is used to pump hydrogen ions (H^+) into the inter-membrane space of the mitochondrion, and the

diffusion of these ions back into the mitochondrial matrix drives production of ATP by ATP-synthase, an enzyme also located on the inner mitochondrial membrane. Simply put, then, food is burned to convert oxygen into water, all to generate energy that cells can use. However, an estimated 1-6% of the molecular oxygen is not reduced to water by the enzyme responsible for doing so (cytochrome oxidase) (Dreosti 1991, Ji 2008). Rather, intermediates of the reduction process escape this enzyme and enter the mitochondrial matrix (Fig. 1.2A). Some of these intermediates are radicals (denoted by a raised dot [$^{\bullet}$] next to an atom); radicals are atoms or molecules with an unpaired electron, and so they readily scavenge electrons from other compounds, damaging them and making them radicals in turn. Highly reactive non-radical compounds that escape the ETC may also undergo reactions that generate radicals. Ignoring their radical or non-radical nature, we call this group of dangerous compounds reactive oxygen species (ROS) or pro-oxidants because they cause oxidation.

If antioxidant enzymes (or other antioxidant compounds) are not available in suitable quantity to convert or quench these reactive intermediates (Fig. 1.2B), they are free to react with (read: "damage") other molecules in the mitochondrion, the cell, and body (depending on how far they, or their products, get). Superoxide ($O_2^{\bullet -}$) and the hydroxyl radical ($^{\bullet}OH$) are particularly reactive free radicals and will quickly take electrons from other molecules (e.g., lipids, proteins, and DNA) to satisfy their unbalanced chemical structure. Meanwhile, hydrogen peroxide is less reactive (not being a radical, but rather an oxidizing acid) and can cross membranes, and hence exert its effects further from the site of production; contact with metals (chiefly iron ions) can convert it to the highly reactive hydroxyl radical (Fig. 1.2C).

Damage to proteins can destroy their structural or enzymatic function, damage to DNA can generate mutations or diminish a mitochondrion's ability to replicate, and damage to lipids can affect membrane structure and function (e.g., decreasing membrane fluidity, affecting permeability, and deactivating membrane-bound proteins; Dreosti 1991). Protection against the damaging effects of these reactive intermediates is, therefore, clearly important.

All of this thus described applies to any vertebrate that uses oxygen-based metabolism to generate energy. Animals performing exercise such as birds during a migratory flight, and those that rely on large amounts of fat as fuel such as migratory birds, face extra complications because: (1) an increase in activity may increase production of ROS in excess of the immediate capacity of antioxidants to quench them, and (2) stored and structural fats, particularly polyunsaturated fatty acids (PUFAs), are especially vulnerable to oxidative damage because of their chemical structure, and may generate their own variety of pro-oxidants.

Why are PUFAs so important to understanding oxidative damage in birds? PUFAs are particularly relevant molecules in the context of bird physiology. We know that birds and mammals differ in the saturation level of fatty acids in their cellular membranes, migratory birds select particular unsaturated fatty acids in their diets, chain length of unsaturated dietary fatty acids can affect exercise performance in birds, and birds appear to be capable of controlling their fatty acid composition in relation to their diet (McWilliams et al. 2004, Costantini 2008, Weber 2009, Pamplona and Costantini 2011, Pierce and McWilliams 2014).

Fatty acids are essential for birds during migration, given that fat is the primary fuel used for long-distance flights, but they are also the foundational components of cellular membranes and therefore crucial to maintaining the integrity and function of cells and cellular compartments. These membranes are particularly vulnerable to oxidative damage for two reasons: (1) their proximity to the site of pro-oxidant production (the membrane-bound ETC), and (2) the ease with which their electrons are stolen by free radicals, especially when double bonds are present. Membrane damage may then cause further damage in stored fats to be used as fuel.

PUFAs are vulnerable to oxidative damage simply because hydrogen atoms located near double bonds are easily oxidized, i.e., they have low oxidative potential, which simply means it takes very little chemical energy to steal them away; hydrogen atoms on carbons between double bonds are particularly vulnerable (Wagner et al. 1994, Milbury and Richer 2008; Fig. 1.3). When an unsaturated fatty acid encounters a free radical like the hydroxyl radical, the fatty acid loses an electron and becomes a lipid radical (Fig. 1.3A); when that lipid radical encounters oxygen, the resultant lipid peroxyl radical (Fig. 1.3B) becomes free to react with another intact PUFA and generate a new radical (Fig. 1.3C). Overall, the entire process can be visualized as a chain reaction (Fig. 1.3D). As shown in Figure 1.3, new lipid radicals can interact with oxygen to generate another lipid peroxyl radical. Thus, one hydroxyl radical can initiate a self-perpetuating chain reaction that needs only the continual input of oxygen and other PUFAs to persist. This is why PUFAs are so vulnerable to ROS, i.e., the reaction will continue either until all PUFAs are oxidized or an antioxidant compound breaks the chain. Vitamin E is an important antioxidant here because it can quench both the hydroxyl radical and the

radicals produced by PUFAs, thereby "breaking the chain" of lipid peroxidation (Dreosti 1991, Milbury and Richer 2008).

The more double bonds present on a fatty acid, the more vulnerable it is to oxidation and the more likely it is to participate in this runaway lipid peroxidation cascade (Wagner et al. 1994). As noted above, damage to these fatty acids changes their properties and function in cells. Equally problematic, however, is their potential to further damage other tissues. Just as by-products of the ETC are called ROS, radicals produced by lipid oxidation are called reactive carbonyl species (RCS), but these RCS pro-oxidant compounds persist much longer in cells than ROS (half-lives of minutes to hours, rather than fractions of a second) and can diffuse through and out of cells, thereby causing damage far from their site of origin (Buttemer et al. 2010). Migrating birds store, mobilize, and burn considerable amounts of fat during their journeys, and ROS and RCS compounds generated in cells can place that fat at risk. As such, for exercising birds with fat stores vulnerable to pro-oxidant attack, protecting PUFAs from damage is important for preserving the integrity of those molecules and their function, as well as preventing the risk of exacerbated damage produced by RCS.

ORGANISM LEVEL: OXIDATIVE STRESS AND HOW TO AVOID IT

What is oxidative stress, really? Although the term "oxidative stress" is often used in the literature, truly quantifying it remains a challenge, and one that is unlikely to be resolved soon. As Costantini (2014) pointed out, "oxidative stress" is really a latent variable, conceptually constructed rather than actually observable because there

is no one metric that defines it. Researchers have provided various definitions of what oxidative "stress" represents, whether it manifests in fitness consequences (Cohen et al. 2010), and how it can be visualized (e.g., seesaw balance of ROS and antioxidant defense, Monaghan et al. 2009; gradient diagrams between extremes of pro-oxidants and antioxidants, Costantini and Verhulst 2009).

For practical design of studies, however, perhaps it is more helpful to think of a bird in terms of the components of its "oxidative status." Take, for example, two scenarios for an exercising (read: migrating) bird (Fig. 1.4A and B). If a bird exercises, producing ROS, and has limited antioxidant capacity (which we can measure in several ways), then a substantial amount of oxidative damage can occur (as indexed by damage to lipids, proteins, and so on; Fig. 1.4A). However, if the antioxidant capacity of the exercising bird is matched to the increased pro-oxidant levels, there may be minimal oxidative damage (Fig. 1.4B). What should be clear in such a system is that we must always at least measure both sides—antioxidants and pro-oxidants, or antioxidants and oxidative damage—to understand the oxidative status of a bird. Unfortunately, there are no direct, field-ready measures of pro-oxidant production. However, there are several useful indicators of oxidative damage and antioxidant capacity (the most popular of which we will discuss below).

Oxidative "stress" then logically manifests itself in some physical or functional consequence of damage. For the purposes of this review, we will define oxidative stress as the accumulation of oxidative damage that affects the performance of a migrating bird. How do migrating birds, therefore, prevent or alleviate the accumulation of such damage?

How do birds avoid oxidative stress? Migrating birds have two main ways of dealing with the heightened threat of oxidative damage during their flights: (1) up-regulate their endogenous antioxidant capacity (e.g., antioxidant enzymes), and (2) consume more dietary antioxidants in preparation for migration and during stopovers (Table 1.1, Fig. 1.4). These are not mutually exclusive. Phenotypic flexibility is a hallmark of both strategies, and discovering which strategy (or combination of strategies) free-living birds adopt in various ecological contexts will enrich our understanding of bird-habitat associations during migration and the parameters that constrain their migratory abilities.

Enzymatic antioxidants, their function, and up-regulation

As shown in Fig. 1.2B, three main varieties of enzymes act as the first line of defense against oxidative damage, given that they intercept the first-escaped by-products of the ETC. Superoxide dismutase (SOD) functions to neutralize the superoxide anion radical ($O_2^{\bullet -}$), whereas glutathione peroxidase (GPX) and catalase (CAT) convert hydrogen peroxide (H_2O_2) to water. GPX is a particularly important scavenger of this compound in animal tissues, and even converts hydroperoxides, such as lipid peroxides produced by the lipid oxidation cascade, into alcohols. GPX relies on the endogenous antioxidant glutathione (GSH) to perform its reactions, and the oxidized form of GSH is consequently recycled for repeated use by the enzyme glutathione reductase.

The utility of SOD, GPX, and CAT extends beyond the inner mitochondrial membrane, however, and various forms of these enzymes may be found in different

cellular compartments. Their activity also tends to vary across tissues, with highest activities in those tissues with the greatest oxygen consumption (Ji 2008). Indeed, a distinct advantage of these enzymes is that they can be up- or down-regulated in response to need, and the effect of physical exertion on their activity levels has been examined in multiple studies (reviewed by Ji 2008). Importantly, however, SOD, GPX, and CAT are not effective in quenching all pro-oxidants or preventing all manner of oxidative damage. In short, these enzymes do not work alone.

Non-enzymatic endogenous antioxidants and their function

We have already mentioned one non-enzymatic endogenous compound important to antioxidant protection, GSH. GSH not only assists GPX, but can also scavenge pro-oxidants; this compound is synthesized from amino acid precursors by glutathione synthetase and, as mentioned above, is converted back to its active form from its oxidized form (glutathione disulfide) by glutathione reductase (McGraw 2011, Costantini 2014). Given that GSH is such an active and important component of the antioxidant system, measurement of the ratio between its reduced and oxidized forms has been used as one indicator of oxidative balance (see "Measuring oxidative balance in a field context" section).

Perhaps one of the best known endogenous antioxidants circulating in birds, however, is uric acid, the final product of nitrogen metabolism in these taxa. It is commonly known that a high-protein diet in birds results in high circulating levels of uric acid (e.g., Alan et al. 2013). Uric acid is a potent scavenger of pro-oxidants, and its level in blood has been used as an indicator of antioxidant capacity. The oxidized

form of uric acid, allantoin, is produced when uric acid reacts with a pro-oxidant, and measurement of the uric acid:allantoin ratio in birds has been proposed as a useful indicator of oxidative balance, as with GSH (Tsahar et al. 2006, McGraw 2011). Unlike GSH, however, uric acid cannot be recycled to its active form once oxidized (Milbury and Richer 2008).

Another compound particularly relevant to endogenous antioxidant protection is the circulating plasma protein albumin. Like GSH and uric acid, albumin scavenges free radicals by being easily oxidized, sparing other molecules by reacting with pro-oxidants itself (Casagrande et al. 2015). Researchers speculate that albumin may protect PUFAs to which it binds, but any oxidized albumin loses its antioxidant capability and must be replaced (Roche et al. 2008).

Circulating hormones may also have antioxidant functions, either as scavengers of pro-oxidants (e.g., estrogen) or as regulators of other endogenous compounds (e.g., glucocorticoids). Melatonin is a hormone with both properties (Pandi-Perumal et al. 2006, McGraw 2011, Beaulieu and Schaefer 2014, Costantini 2014). Given that melatonin is secreted chiefly at night, the potential importance of this hormone for nocturnally migrating birds is high and deserves further study.

Lastly, it is important to acknowledge the endogenous compounds that help prevent conversion of hydrogen peroxide to the hydroxyl radical by sequestering the metals that catalyze that reaction. Ferritin and ceruloplasmin are two examples of molecules that chelate free metal ions so that they cannot react with peroxides and therefore facilitate oxidative damage (Costantini 2014).

Dietary antioxidants, their origin, function, and interactions

Although enzymes and other endogenous antioxidant compounds must be synthesized by cells in response to demand, dietary antioxidants can be acquired through feeding and may therefore be a "cheaper" alternative (in terms of both time and energy) if they can supplement or replace the action of endogenous components of the antioxidant system (Pamplona and Costantini 2011). Endogenous and exogenous antioxidants are not broadly interchangeable, but evidence suggests that supplementation of some dietary antioxidants may result in lower activity of antioxidant enzymes with no concomitant change in total antioxidant protection (Costantini 2014). Broadly speaking, the benefits of dietary antioxidants span a range of protective mechanisms, from scavenging pro-oxidants such as the hydroxyl radical, to chelating metals, strengthening the immune system, and stimulating the up-regulation of endogenous antioxidants (Lushchak 2011, Costantini 2014). Dietary antioxidants are typically classified as either water-soluble (hydrophilic) or fat-soluble (lipophilic), and therefore may be distributed, stored, or mobilized in various tissues depending on their solubility.

Dietary antioxidants used by animals originate in photosynthetic plants where they act as pigments and protect plant tissues from sunlight, the radicals generated during photosynthesis, oxidation from the air, and reactive by-products of oxygen metabolism. Thus, plants produce and use antioxidant compounds to protect their own cells and invest them in tissues, e.g., leaves, nectar, seeds, and fruits, and animals gain protection by ingesting them. Antioxidants in edible plant parts may have originated to protect those tissues, but they also appear to offer a nutritional reward to birds that eat

fruits and consequently scatter the seeds. That is, plants use birds just as the birds use plants (Izhaki and Safriel 1985, Smith and McWilliams 2014a). Seeds and fruits may vary widely in their total antioxidant content, and particular constitutive compounds, and birds may choose these foods based on their antioxidant content (e.g., Schaefer et al. 2008, Alan et al. 2013, Bolser et al. 2013). Several main classes of dietary antioxidant are particularly relevant to bird studies, including carotenoids, vitamin E, and polyphenols.

Carotenoids represent a large group of chiefly orange, red, and yellow pigments with antioxidant properties. Specifically, they are capable of scavenging hydroxyl and other radicals, becoming relatively stable radicals themselves in the process. Although their contribution to circulating antioxidant capacity has been estimated to be quite low (Costantini and Møller 2008, Simons et al. 2012), their value out of circulation should not be ignored (Cohen and McGraw 2009); carotenoids are fat-soluble, rapidly absorbed, and therefore readily integrated into cellular membranes and lipid-dense tissues, offering them antioxidant protection. As pigments, they are responsible for the color of furcular fat deposits and egg yolks, as well as the sexual ornaments of many birds. Carotenoids may be mobilized from fat deposits at time of need (Metzger and Bairlein 2011), they contribute to the survivability of growing embryos and hatchlings (McGraw et al. 2005), and carotenoids may even help protect muscles during flight exercise or otherwise enhance flight performance (Blount and Matheson 2006, Mateos-Gonzalez et al. 2014). Lutein and its isomer, zeaxanthin, are two of the most common carotenoids in plants (Shahidi and Ho 2007), and therefore of

primary relevance to bird studies (Koutsos et al. 2003, McGraw et al. 2005, Casagrande et al. 2015).

Vitamin E also represents a group of molecules with common structures and functions. Like carotenoids, vitamin E is fat-soluble and therefore serves its primary protective function in fatty tissues, e.g., cellular membranes, fat deposits, and egg yolk. More than any other dietary antioxidant, vitamin E is considered key to protecting lipids from oxidative damage, given its ability to break the chain reaction of the lipid oxidation cascade (Fig. 1.3; Cohen and McGraw 2009, Teixeira et al. 2009, Pamplona and Costantini 2011, Costantini 2014). Specifically, vitamin E can react with lipid peroxyl radicals, preventing further radicalization of additional fatty acids and becoming a relatively stable radical itself in the process. Alpha-tocopherol is the major form of vitamin E typically examined in bird studies, and has been widely studied for its benefits to domestic poultry (e.g., Surai 2002).

So far we have mentioned two major groups of fat-soluble antioxidants, both of which have been well studied in birds. An emerging area of research now focuses on an enormous group of water-soluble, plant-derived compounds, the polyphenols. The subset of polyphenols most relevant to our discussion is the flavonoids, which include the anthocyanins—itself a class of > 200 sugar-bound pigmented compounds (Shahidi and Ho 2007). Flavonoids are the most ubiquitous and potent (at least *in vitro*) antioxidants in nature, appearing in high concentrations in vegetables and fruits, including those that birds choose to consume during migration (Schaefer et al. 2008, Alan et al. 2013, Bolser et al. 2013). In plant tissues, flavonoids serve various functions, including protecting cells against UV radiation and scavenging pro-oxidants

generated during photosynthesis (Pietta 2000). Pigmented under acidic conditions, anthocyanins specifically are responsible for the dark blue, red, and purple colors present in many fruits, and birds appear to use this coloration to distinguish the antioxidant content of those fruits (Schaefer et al. 2008). In the bodies of animals, polyphenols display a variety of beneficial functions, scavenging pro-oxidants, including in the digestive system where they may protect ingested food from oxidation (Pietta 2000, He et al. 2006), facilitating the mounting of an immune response (Catoni et al. 2008), chelating metal ions and preventing them from participating in reactions that generate pro-oxidants (Pietta 2000), and up-regulating the expression of antioxidant enzymes and production of GSH (Moskaug et al. 2005, Spanier et al. 2009, Yeh et al. 2009).

Debate has arisen concerning the extent to which polyphenols can be absorbed and stored in the tissues of birds and other animals, but recent studies provide convincing evidence that they are indeed metabolized and available for an appreciable period of time after ingestion. Over a decade ago, Cao and Prior (1999) showed for the first time that anthocyanins can be absorbed in their sugar-bound form in humans, and they are measurable in the circulation for at least 1 h. Catoni et al. (2008) demonstrated that European Blackcaps (*Sylvia atricapilla*) absorb dietary flavonoids and circulate them in blood, and Beaulieu and Schaefer (2014) showed that Gouldian Finches (*Erythrura gouldiae*) circulate dietary polyphenol metabolites for at least several hours.

Furthermore, polyphenol compounds may undergo several chemical changes during digestion (removal of associated sugar, conversion to a phenolic acid) that

nevertheless do not diminish their antioxidant potency, and their chemical properties (type of associated sugar, methylation) may affect how they are integrated into tissue (Pietta 2000, He et al. 2006). Clearly, there is ample opportunity to examine how birds choose dietary polyphenols in foods (as first demonstrated by Catoni et al. 2008 and Schaefer et al. 2008) and how they may alter their structure to utilize them *in vivo*.

A complex interacting system

All of the antioxidants we have discussed so far have unique chemical structures, solubilities, scavenging properties, and capabilities, and none of them act exclusively alone. Rather, research is continuously revealing how multiple antioxidants act in concert, and deficiencies in one can lower the efficacy of others. Although not often a sole focus of wild bird research, vitamin C (ascorbic acid) is another potent dietary antioxidant of note, mainly because of its interactions with other antioxidants, particularly vitamin E.

Before we discuss how these antioxidants interact, understanding why they do so is important. Whether or not a molecule can be (or will be) oxidized by another molecule comes down to comparing the "oxidation potential"—the amount of energy required to remove yet another electron from the first molecule. These oxidation potentials explain why vitamin E (alpha-tocopherol) protects PUFAs from oxidative damage because an electron on the vitamin E molecule is stolen much more readily than, i.e., has a lower oxidation potential than, an electron on a PUFA. All things being equal, a hydroxyl radical will steal an electron from vitamin E before it steals one from a PUFA, leaving the PUFA intact.

Vitamin E molecules become oxidized when they serve their protective function and therefore lose their antioxidant properties unless they recover an electron. This is where other antioxidants come in; vitamin C has an even lower oxidation potential than vitamin E and can "restore" vitamin E to its protective form. Vitamin C then itself becomes a radical, but can be "restored" by reduced GSH, which has an even lower oxidation potential and is easily recycled itself (when oxidized to oxidized GSH, GSSG) by the enzyme glutathione reductase (Milbury and Richer 2008).

Because of its very low oxidation potential, reduced GSH can be easily oxidized by pro-oxidants, sparing other molecules from oxidation or restoring other recyclable antioxidants, and is, therefore, a very important endogenous antioxidant (Milbury and Richer 2008). Additionally, polyphenols may help maintain high levels of vitamin E in membranes by protecting that important antioxidant from oxidation and indirectly protecting lipids despite their water-solubility (Pietta and Simonetti 1998, Pietta 2000); furthermore, some oxidized polyphenols may rely on regeneration from vitamin C and GSH (Pietta 2000). Clearly, circulating non-enzymatic antioxidants interact to prevent oxidative damage in animals.

ECOLOGICAL LEVEL: OXIDATIVE BALANCE AS A MIGRATORY CONSTRAINT

Oxidative damage occurs at the atomic level and birds do have ways to prevent damage on an organismal level, but how does oxidative balance relate to avian life history and patterns/processes at the ecological level? We would like to make the case that considering oxidative balance is crucially important to understanding several

aspects of the behavioral choices of birds and their distribution during migration. In particular, we posit that oxidative balance is relevant to thinking about (1) flight distance and migration strategy and (2) nutritional choices at stopover sites.

How does flight exercise affect oxidative balance in birds? Given the demands of intense flight exercise, migrating birds should seek to minimize the potential for oxidative stress during each flight bout, whether by reducing the time spent aloft, consuming prophylactic antioxidants, up-regulating endogenous antioxidants, repairing damage after landing, or a combination of all of these strategies. Because of several pioneering studies, we know that oxidative damage is a real hazard of flight by volant birds, both domesticated and free-living (Costantini et al. 2007, 2008, Jenni-Eiermann et al. 2014). However, we also know that birds can prepare for and repair such damage (Jenni-Eiermann et al. 2014, Skrip et al. 2015).

Costantini et al. (2008) provided some of the first direct evidence that flying causes oxidative damage in birds. They flew trained pigeons for short (60 km) and long durations (200 km) and took blood samples within 15 min after flights to acutely measure oxidative damage and antioxidant capacity. Pigeons that flew for 200 km exhibited a 54% increase in oxidative damage (as measured by serum reactive oxygen metabolites [ROMs]), and a 19% decrease in total serum non-enzymatic antioxidant capacity (measured by the OXY-adsorbent test), presumably because some of the antioxidant capacity was used to quench pro-oxidants, although not enough to avoid oxidative damage.

Subsequently, Jenni-Eiermann et al. (2014) examined European Robins (*Erithacus rubecula*) captured in flight at night as they migrated through a Swiss

mountain pass. Both a marker of oxidative damage (protein carbonyls) and a marker of enzymatic antioxidant capacity (glutathione peroxidase) were significantly higher in birds captured during the night than in conspecifics captured while on stopover during the day, presumably because flight caused muscle damage and the antioxidant system was up-regulated during migration. These researchers further showed that antioxidant enzyme levels were higher in after-hatch-year birds than hatch years, suggesting that experienced animals were better able to prepare for the oxidative challenge.

The results of laboratory studies also suggest that animals can up-regulate their own endogenous defenses in response to exercise training, decreasing levels of oxidative damage and even compensating for an antioxidant-deficient diet (Teixeira et al. 2009, Janiak et al. 2010, Larcombe et al. 2010). Oxidative damage in the plasma of adult Budgerigars (*Melopsittacus undulatus*) can be reduced by exercise training regardless of antioxidant levels in the diet, indicating that endogenous antioxidants are up-regulated by repeated flight bouts (Larcombe et al. 2010). In horses, long-duration training programs for distance racing can increase levels of antioxidant enzymes (particularly GPX) in red blood cells (Janiak et al. 2010). Furthermore, oxidative damage is highest in the tissues (e.g., liver, heart, and skeletal muscle) of rats fed an antioxidant-deficient diet and not exercised, but low and equivalent in exercised rats on either a deficient or standard diet (Teixeira et al. 2009).

Regardless of whether a bird increases its antioxidant capacity for migration, some damage will inevitably occur. Birds appear, however, to be able to readily remove or repair damaged molecules. Jenni-Eiermann (2014) showed that European Robins resting during the day exhibited lower protein damage levels than those

actively migrating, and Skrip et al. (2015) showed that lipid peroxidation levels in Garden Warblers (*Sylvia borin*) decreased with time on stopover after spring migration over the Sahara Desert and Mediterranean Sea. The ability of birds to recover from damage, and prepare for the demands of further flights, likely depends heavily on their use of stopover sites. Although not yet explicitly tested at a large scale, the arrangement of stopover sites on the landscape may dictate not only how birds can refuel, but how they can cope with oxidative damage.

How do stopover sites help birds manage oxidative balance? During migration, stopover sites provide opportunities for birds to rest, refuel on fats and protein, acquire water, and potentially to recover from accumulated oxidative damage, but they also offer, especially during fall migration, seasonally abundant fruits rich in antioxidants. An opportune area of future research concerns the use of stopover sites for building non-enzymatic antioxidant capacity in concert with energy stores, particularly through dietary choices (Skrip et al. 2015).

Many insect-eating and seed-eating birds shift to eating fruits during fall migration (Parrish 1997), and this "diet-switching" has been widely understood as a common strategy by many species to use nutritious, and seasonally abundant, fruits to quickly and efficiently build fat stores. Building fat stores of good quality in a short time is necessary for animals that must burn large amounts of high-energy fuel to complete their migrations (McWilliams et al. 2004). Studies show that fruit-rich stopover habitats produce birds with higher fattening signatures (circulating triglycerides) in their blood than similar, but fruit-poor, habitats (Smith and

McWilliams 2010). However, birds may also consume fruits for additional nutritional benefits.

Although migrating birds can be expected to select foods with high energy density—and they do—antioxidant content may also influence their decisions. Research has shown that birds are able to discriminate among fruits based on their color, and hence anthocyanin content, and prefer anthocyanin-enriched foods when presented a choice (Schaefer et al. 2008). On Block Island, a stopover site for millions of songbirds each autumn off the coast of Rhode Island, USA, arrowwood viburnum (*Viburnum dentatum*) fruits are highly prized by birds, presumably due to their unique combination of abundant, high-quality fats and both fat- and water-soluble antioxidants (Smith et al. 2007, 2015, Alan et al. 2013, Bolser et al. 2013). Influxes of migratory birds to Block Island during peaks in fall migration are regularly followed by increases in rates of arrowwood removal (Smith and McWilliams 2014a). Even in years of low arrowwood fruit yield, birds seek and choose to consume this species at a greater rate than fruits of other species that are more abundant, suggesting the importance of this shrub to the diet of migrating birds in this region (Bolser et al. 2013).

Several authors have speculated that birds may face trade-offs while feeding, depending on whether their needs are greater for energy or for particular compounds such as antioxidants (Monaghan et al. 2009, Beaulieu and Schaefer 2013, 2014). However, these conceptual trade-offs may not exist when birds consume fruits that are high in both energy density and antioxidants. Birds on Block Island show strong correlations between fat stores and circulating non-enzymatic antioxidants, suggesting

that they may acquire both in their diet as they prepare for migration (Skrip et al. 2015). In Europe, researchers have also found anthocyanin and caloric content to be positively correlated in autumn fruits (Schaefer et al. 2008).

The type of antioxidants in high-fat fruits may also be particularly advantageous for consumers, given that high-fat fruits should contain antioxidants that specifically protect fats from spoiling before consumption by animal dispersers. For example, arrowwood fruits on Block Island contain abundant water-soluble polyphenols (the many benefits of which were discussed above), and most of their fat-soluble antioxidants are represented by vitamin E (Alan et al. 2013), the benefits of which may be twofold: protecting lipids in the fruits against oxidation, and conferring valuable protection to the fat depots and membranes of migrating birds that rely heavily on fat metabolism.

Especially when abundant in foods that offer other nutritional benefits, dietary antioxidants may be considered relatively "inexpensive" to acquire and use (Beaulieu and Schaefer 2013). Oxidative damage may occur because endogenous defenses take time, energy, and molecular resources to up-regulate, and so organisms can take advantage of dietary antioxidants to compensate. Dietary or non-enzymatic antioxidants may be most essential to animals not accustomed to long-distance exercise, e.g., birds making a migratory flight for the first time or early in the migration season (Costantini 2008, Larcombe et al. 2010, Beaulieu and Schaefer 2013). How birds may strategically use these antioxidants, however, still remains an open question. Beaulieu and Schaefer (2013), in particular, speculated that migrating birds may use specific antioxidants prophylactically, in anticipation of impending

need, and may take advantage of a series of stopover sites, or a combination of fat- and water-soluble antioxidants that may or may not be stored in tissue, during the course of their migrations. Their logical arguments now require empirical evidence (e.g., Skrip et al. 2015).

MEASURING OXIDATIVE BALANCE IN A FIELD CONTEXT

Now that we have covered the basics of oxidative challenges in migrating birds, we can provide some recommended field measurements for oxidative damage and antioxidant capacity that ornithologists can include in their tool kit of techniques (Table 1.2). As hopefully is clear from Figure 1.4, measuring multiple aspects of an animal's oxidative status, i.e., both antioxidant capacity and damage, is important for understanding how a bird is coping with an oxidative challenge, and so we highlight ways of evaluating both.

As described above, different antioxidant compounds work in different ways, and oxidative damage may be represented by multiple indicators (e.g., protein or fat). Antioxidants may also be concentrated or most active in different sites in the body, e.g., fatty tissue, muscle, egg, plasma, or red blood cells. Choosing the most appropriate metric (and tissue) for a research question is also important. We acknowledge that many researchers will prefer to sample blood as a non-invasive, non-lethal way to answer different questions, especially field ornithologists sampling protected species, and so we emphasize here what can be measured in blood. For researchers new to sampling avian blood, we highly recommend reading Owen (2011).

We should also make clear that our intention is not to provide an exhaustive treatment of all of the measures that can be used to gauge oxidative status, or their various uses. For those interested in additional information, we recommend Urso and Clarkson (2003), Hõrak and Cohen (2010), and Halliwell and Gutteridge (2015). In addition, Cohen et al. (2007), Cohen and McGraw (2009), and Cohen et al. (2009) provide additional applications. Here, we highlight some commonly used measures that are most practical for field studies (Table 1.2).

Measuring antioxidant capacity in blood. Emerging research suggests that multiple components (enzymatic or non-enzymatic) of the antioxidant system of birds may co-vary or operate independently—depending on the components—and so there may be animal-wide integration or compartmentalization of antioxidant responses (Costantini et al. 2011, 2013). Therefore, in general, we recommend pairing a measure of non-enzymatic antioxidant capacity in plasma and/or red blood cells with a measure of antioxidant enzyme activity (chiefly GPX) in red blood cells for questions that require a broad, whole-animal gauge of antioxidant levels. We recommend further measurement of individual antioxidant compounds for questions that address dietary effects on circulating antioxidant levels or questions targeted to the effects of oxidative challenges on particular aspects of the antioxidant system.

Total non-enzymatic antioxidant capacity

There are several general assays that can be used to assess the capacity of the non-enzymatic antioxidant pool of a plasma sample to quench pro-oxidants, e.g., OXY-adsorbent test (OXY), Trolox equivalent antioxidant capacity (TEAC; also

known as total antioxidant capacity [TAC]), ferric reducing ability of plasma (FRAP), and oxygen radical absorbance capacity (ORAC; Monaghan et al. 2009, Costantini 2011). These assays, however, do not measure the same components, and their usefulness under different circumstances has been discussed elsewhere (Costantini 2011, Alan and McWilliams 2013).

Two spectrophotometric assays that have been used widely and are relevant for field studies are TEAC and OXY (Table 1.2). As modified by Cohen et al. (2007), the TEAC test can be run on small volumes of blood and accounts for a variety of circulating micromolecular antioxidants, excluding albumin and other proteins. TEAC works by activation of a chromogenic free radical by hydrogen peroxide. Antioxidants in the sample being tested quench this radical to convert it back to its clear form. Recently, however, avian studies—including our own—favored use of the OXY test, a commercial kit (Diacron International, Grosseto, Italy) that measures the ability of a plasma sample to neutralize an oxidizing assault of hypochlorous acid (HOCl), an endogenous pro-oxidant. Like TEAC, this test accounts for a variety of non-enzymatic antioxidants, including vitamins C and E, carotenoids, and thiols (Costantini 2011), and can be run on small volumes of plasma.

A main difference between the two tests is whether they include the action of uric acid, and hence must be corrected for it. Although non-enzymatic endogenous antioxidants like GSH are up- or down-regulated in response to need, and exogenous antioxidants such as vitamin E and carotenoids are acquired through the diet, uric acid is a catabolically derived antioxidant; it is the final product of protein catabolism in birds and, therefore, its increased presence in the circulation is the result of increased

breakdown of dietary or body protein. Whether an increase or decrease in circulating uric acid can reliably indicate a bird's preparation for or response to an oxidative challenge is therefore unclear. Researchers often prefer to evaluate uric acid separately, given that its origin and function differ from those of other antioxidants.

The different chemical processes of assays dictate what they measure. The TEAC test measures the contribution of uric acid to circulating antioxidant capacity and so uric acid is typically measured along with TEAC in a separate assay; a simple linear regression analysis can then yield "residual TEAC" values used to evaluate the non-uric acid portion of total antioxidant capacity (e.g., Cohen et al. 2007, Cohen and McGraw 2009, Alan and McWilliams 2013, Cram et al. 2015). OXY does not include the antioxidant properties of uric acid, so is useful for studies where investigators do not want to integrate this potentially confounding contribution into their measure of antioxidant capacity (Costantini 2011, Alan et al. 2013).

Enzymatic protection of red blood cells

Pairing measurement of non-enzymatic antioxidant capacity of plasma with measurement of GPX in red blood cells (Table 1.2) is emerging as an important research strategy because the non-enzymatic and enzymatic portions of the antioxidant system appear to work in concert through different mechanisms (Costantini et al. 2011). Costantini et al. (2011: 1151–1152) suggested that the two systems may be regulated differently, and specifically suggested that "measuring both antioxidant capacity and GPX activity may produce a better estimate of blood antioxidant status than measurements of a single component." Remember that GPX is specifically useful

in neutralizing hydrogen peroxide and other hydroperoxides, including lipid peroxides from the lipid peroxidation cascade; this enzyme therefore appears to be particularly important in protecting red blood cells from oxidative damage from these pro-oxidants (Jenni-Eiermann et al. 2014). We suggest that this aspect of antioxidant capacity be favored in future studies as an indicator of the acute endogenous responses of birds to oxidative challenges, along with erythrocyte activities of SOD and CAT if sample volumes permit (e.g., Oropesa et al. 2013). Measuring GPX in red blood cells typically involves the spectrometric Ransel assay, where the activity of this enzyme and its ability to oxidize GSH is assessed by a decrease in absorbance (Costantini et al. 2011, 2013, Jenni-Eiermann et al. 2014).

Specific circulating antioxidants

The antioxidants most commonly measured in the circulation of birds include uric acid, GSH and other thiols (compounds with a sulfhydryl [–SH] group), vitamin E, carotenoids, and polyphenols (Table 1.2). Although uric acid, GSH, and other thiols are endogenously produced and therefore their levels can be internally modified, vitamin E, carotenoids, and polyphenols are acquired by birds through their diets. The choice to track individual antioxidants, therefore, depends on the research question. For example, uric acid may be measured specifically in studies focused on dietary or muscular protein catabolism, whereas vitamin E may be measured and tracked during supplementation experiments, and carotenoids or polyphenols may be assayed during feeding studies, studies that track how these compounds are assimilated and/or

mobilized, and/or studies that link antioxidant capacity with dietary habits on stopover.

As discussed above, uric acid has antioxidant properties in addition to serving as the final, excreted, product of protein metabolism. Researchers may find measuring this antioxidant most useful in contexts where they expect protein (whether dietary or from muscle) to be metabolized in large amounts during an oxidative challenge (e.g., Alan and McWilliams 2013). Although uric acid is an antioxidant in its reduced form, the ratio of uric acid and its oxidized form, allantoin, has also been suggested to be an indicator of oxidative status (see Tsahar et al. 2006 for some methodological considerations for measurement). Birds lack the enzyme urate oxidase, and therefore the presence of allantoin in these species indicates non-enzymatic oxidation of uric acid—in other words, an oxidative challenge (Tsahar et al. 2006).

The benefits of GSH were noted previously, particularly as a substrate for the enzyme GPX; circulating levels of GSH and GPX seem to be integrated (Costantini et al. 2011). Measuring the ratio between the reduced (helpful) form of GSH and its oxidized form (GSSG) in particular may provide a useful index of oxidative status, a concept similar to the ratio of reduced-to-oxidized uric acid. This ratio between GSH and GSSG has been examined in relation to exercise in humans (see summary and citations in Urso and Clarkson 2003) and in the context of toxicant challenges in birds (Henny et al. 2002, Hoffman 2002, Isaksson et al. 2005). Its applicability to avian reproductive and migratory contexts deserves further study.

Vitamin E, carotenoids, and polyphenols (including anthocyanins and related compounds) can be measured in plasma via high performance liquid chromatography

(HPLC) (vitamin E and carotenoids: Cohen and McGraw 2009, Cohen et al. 2009; polyphenols: Cao and Prior 1999, He et al. 2006, Catoni et al. 2008). Given the increasing recognition of anthocyanins in the diet of birds during migration (Alan et al. 2013, Bolser et al. 2013), we believe future researchers would benefit from tracking these compounds in wild birds on stopover, particularly when fruit consumption is high. Remember, however, that anthocyanins, and other flavonoids, may be degraded to other metabolites during digestion, and so researchers may benefit by looking for not only the original compounds in blood, but also derivative metabolites. Furthermore, there is evidence that carotenoids are stored in, and mobilized from, fatty tissues and may not contribute highly to circulating antioxidant capacity in a chronic way (Costantini and Møller 2008, Cohen and McGraw 2009, Metzger and Bairlein 2011, Simons et al. 2012); therefore, these measures may be most helpful for tracking the acute accumulation or mobilization of dietary antioxidants at stopover sites or during oxidative challenges.

Measuring oxidative damage in blood. Measures of oxidative damage are as varied as measures of antioxidants, and can provide different glimpses into a bird's oxidative status. For birds during migration, the most relevant markers are those for lipid and protein oxidation. Wherever possible, we recommend that these two types be used in concert.

Markers of lipid oxidation

Given the importance of fats to migrating birds, measuring lipid damage will be important for gauging how exercise and other stressors affect their oxidative status.

Lipid oxidation can be assessed in various forms, e.g., isoprostane assays, detection of malondialdehyde (MDA) via HPLC, thiobarbituric acid reactive substances (TBARS), or d-ROMs test (Monaghan et al. 2009, Cohen et al. 2010). A prospective problem with all of these assays is the potential for detection of confounding molecules, inflating estimates of natural damage (Monaghan et al. 2009).

The d-ROMs test kit (Diacron International, Grosseto, Italy; Table 1.2) has emerged as a favored assay in avian studies (e.g., Costantini et al. 2007, 2008, 2011, 2013, 2014, 2015, Alan and McWilliams 2013, Skrip et al. 2015). The test measures oxidative damage as the presence of circulating hydroperoxides, which include products of the lipid oxidation cascade, as well as protein and nucleic acid oxidation. Kilk et al. (2014) suggested that the copper-containing protein ceruloplasmin may contribute to test signal, but Costantini et al. (2014, 2015) found otherwise. The d-ROMs test has revealed many useful results regarding the oxidative status of exercising birds including, e.g., higher d-ROMs levels in exercised pigeons (Costantini et al. 2008), a decrease in levels of d-ROMs the longer birds were on stopover after flights (Skrip et al. 2015), and d-ROMs values predicted return of seabirds to their nesting sites in multiple breeding seasons (Costantini and Dell'Omo 2015). In addition, circulating levels of d-ROMs co-varied with circulating triglyceride levels in birds (Pérez-Rodríguez et al. 2015) and were affected by the fat quality in the diets of birds (Alan and McWilliams 2013). Furthermore, we have found that d-ROMs are correlated with fat stores in migratory birds on stopover both before and after long flights (Skrip et al. 2015).

Markers of protein oxidation

In addition to fats, protein is also essential for migration performance, not only to support fat-based metabolism, but also as a source of metabolic water (Gerson and Guglielmo 2011) and to restore flight and other muscles. Reactions with pro-oxidants can introduce carbonyl groups (an oxygen double bonded to carbon [C = O]) into proteins, and these stable changes are easily detected in plasma (e.g., Costantini et al. 2013) and red blood cells (e.g., Jenni-Eiermann et al. 2014). Protein carbonyls (Table 1.2) are commonly accepted as a useful indicator of protein damage from oxidation, and can be measured via colorimetric assay while accounting for total protein in samples, including via use of commercial kits (e.g., Heiss and Schoech 2012). Recently, high levels of this protein damage have been associated with low muscle score in actively migrating songbirds, suggesting that individuals in poor condition may suffer more oxidative damage (Jenni-Eiermann et al. 2014). The effect of diet and flight exercise on protein damage deserves further study, particularly in the context of preparation for and recovery from long-duration flights.

FUTURE DIRECTIONS

Condition-dependent aspects of oxidative state in birds during migration. Recent work during autumn migration has shown the circulating non-enzymatic antioxidant capacity of birds preparing for long flights to be condition-dependent, with fatter birds showing higher antioxidant capacity (Skrip et al. 2015). The mechanisms underlying this phenomenon have yet to be investigated. Particularly revealing would be the studies that manipulate fat condition of migrants (e.g., Smith and McWilliams

2014b) and/or track fattening rates in birds using plasma metabolites such as triglycerides (e.g., see Smith and McWilliams 2010), and then also measure antioxidant capacity, damage, or particular antioxidants (e.g., Pérez-Rodriguez et al. 2015). Partitioning blood sampling to focus on dawn, midday, and evening feeding, when hungers for different requirements are expected to differ (e.g., Beaulieu and Schaefer 2014), would also be most illuminating. In addition, future researchers may focus on how migration distance, protein content of diet, and stopover duration (and hence extent of damage repair) affects damage levels in wild birds.

Availability of dietary antioxidants during migration. Thus far, research concerning dietary antioxidants on stopover has focused on autumn migration in temperate regions. Whether and how birds acquire dietary antioxidants before their spring journeys from tropical areas remains a considerable gap in our understanding of migratory birds. Furthermore, for both seasons, we have yet to fully understand how use of stopover sites helps birds overcome oxidative costs. How does the specific mixture of antioxidants in bird blood and fat depots change within and between stopovers? What is the effect of flight on levels of particular antioxidant compounds, and does diet dictate the mixture? The distribution and seasonal availability of antioxidant- or calorie-rich plant communities could shape the migration strategies of songbirds, and so learning how birds choose and utilize foods on the landscape may aid conservation efforts.

Processing and storage of dietary antioxidants. It is well established that birds are capable of assimilating dietary antioxidants, and that fat-soluble antioxidants such as carotenoids and vitamin E can be stored in tissues, but a major gap in our

understanding concerns the storage and use of water-soluble antioxidants such as polyphenols, and how the processing and routing of dietary antioxidants prevent damage in the most vulnerable tissues. If birds eat antioxidants, do they make it to the mitochondria where pro-oxidants are generated? If there is greater demand in some tissues for antioxidants (e.g., flight muscle in exercising birds) does the routing of antioxidants change? Given that antioxidants interact and recycle each other (see above), do volant birds manage combinations of antioxidants differently compared to non-flying vertebrates?

Carry-over effects and competing demands. Studies that evaluate the oxidative condition of migratory birds on breeding grounds will be instrumental in teasing apart the costs of oxidative damage, e.g., whether effects of flight (and inadequate preparation or recovery) are immediate and brief, and whether they have fitness costs (e.g., Costantini and Dell'Omo 2015). Given that birds begin breeding in spring shortly after performing challenging long-distance flights, how do the competing demands of reproduction versus migration influence allocation of antioxidants? Are wild birds with higher antioxidant levels before and after migration better able to provision their eggs with antioxidants, or suffer less oxidative damage during chick-rearing?

Measurement of acute change and marker sensitivity to external change. Further research on the utility of different oxidative markers for various study questions, and their sensitivity to confounding factors such as handling, time since capture, and time of day will help improve study design. For example, in several studies, investigators have failed to detect directional changes in OXY and d-ROMs

between first capture and restraint in a cloth handling bag for between 20 and 162 min (Costantini et al. 2007, Skrip et al. 2015). However, researchers have yet to determine if acute changes occur within a 20-min period or confirm whether time of day is an important confounding factor for evaluation of oxidative status. Further methodological research will be of utmost importance in evaluating the oxidative state of birds in field contexts.

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Table 1.1. Summary of antioxidants mentioned in text.

Endogenous antioxidants (i.e., those produced by birds)		Exogenous antioxidants (i.e., those eaten by birds)	
Enzymatic		Fat-soluble (lipophilic)	
Superoxide dismutase (SOD)	First line of defense, neutralizes the superoxide anion radical (O2•-), generating hydrogen peroxide (H2O2)	**Carotenoids**	Large group of chiefly orange, red, and yellow pigments with antioxidant properties
Glutathione peroxidase (GPX)	First line of defense, converts hydrogen peroxide to water and lipid peroxides to alcohols	**Vitamin E**	Large group of antioxidants chiefly responsible for preventing lipid oxidation; alpha-tocopherol is the most common/studied form
Catalase (CAT)	First line of defense, converts hydrogen peroxide to water		
Non-enzymatic		Water-soluble (hydrophilic)	
Glutathione	Neutralizes radicals independently, recycles other antioxidants, assists GPX, is recycled to reduced form by glutathione reductase	**Polyphenols**	Large group of compounds with antioxidant (and other) properties, includes the anthocyanins (red, blue, purple pigments)
Uric acid	Catabolically derived as final waste product of protein metabolism, with antioxidant properties		
Albumin	A circulating protein that scavenges free radicals	**Vitamin C**	Compound that supports immune function and has antioxidant properties, can recycle oxidized vitamin E back to its reduced form
Estrogen and melatonin	Circulating hormones with antioxidant properties		
Ferritin and ceruloplasmin	Proteins that sequester metal ions that might otherwise react with molecules to produce pro-oxidants		

Table 1.2. Recommended methods for measuring the oxidative status of birds using blood samples.

Measure	Method	Helpful methodological references
Antioxidant capacity		
Total non-enzymatic antioxidant capacity		
TEAC	Colorimetric assay to measure ability of sample to reduce radicals formed by reaction with hydrogen peroxide	Cohen et al. (2007)
OXY-adsorbent test	Commercial kit; colorimetric assay to measure ability of sample to neutralize hypochlorous acid	Costantini (2011); Costantini et al. (2011)
Enzymatic protection of red blood cells		
GPX	Commercial kit; kinetic colorimetric assay to measure activity of GPX	Costantini et al. (2011, 2013), Jenni-Eiermann et al. (2014)
Specific circulating antioxidants		
Uric acid	Many colorimetric commercial kits are available to measure uric acid in circulation	Tsahar et al. (2006), Cohen et al. (2007), Costantini (2011)
Uric acid : allantoin ratio	Uric acid and allantoin colorimentric assays run separately to compare values of reduced and oxidized forms	Tsahar et al. (2006)
Glutathione and other thiols	Commercial kit, colorimetric assay to measure compounds with sulfhydryl (–SH) groups that can reduce pro-oxidants	Costantini et al. (2011, 2013)

GSH: GSSG ratio	Colorimetric determination of reduced and oxidized glutathione, with or without a commercial kit	Mahmoud and Edens (2003), Isaksson et al. (2005)
Vitamin E	Detection of compound-specific peak via HPLC	Supporting Information (Appendix 4) in Cohen and McGraw (2009), Online Supplementary Material in Cohen et al. (2009)
Carotenoids	Detection of compound-specific peak via HPLC	Supporting Information (Appendix 4) in Cohen and McGraw (2009), Online Supplementary Material in Cohen et al. (2009)
Polyphenols	Detection of compound-specific peak via HPLC	Cao and Prior (1999), He et al. (2006), Catoni et al. (2008)
Oxidative damage		
Markers of lipid oxidation		
d-ROMs test	Commercial kit; colorimetric assay to measure hydroperoxides	Costantini et al. (2007, 2011, 2013)
Markers of protein oxidation		
Protein carbonyls	Commercial kit is available although not always used; colorimetric assay to detect carbonyl groups per unit protein	Costantini et al. (2013), Jenni-Eiermann et al. (2014)

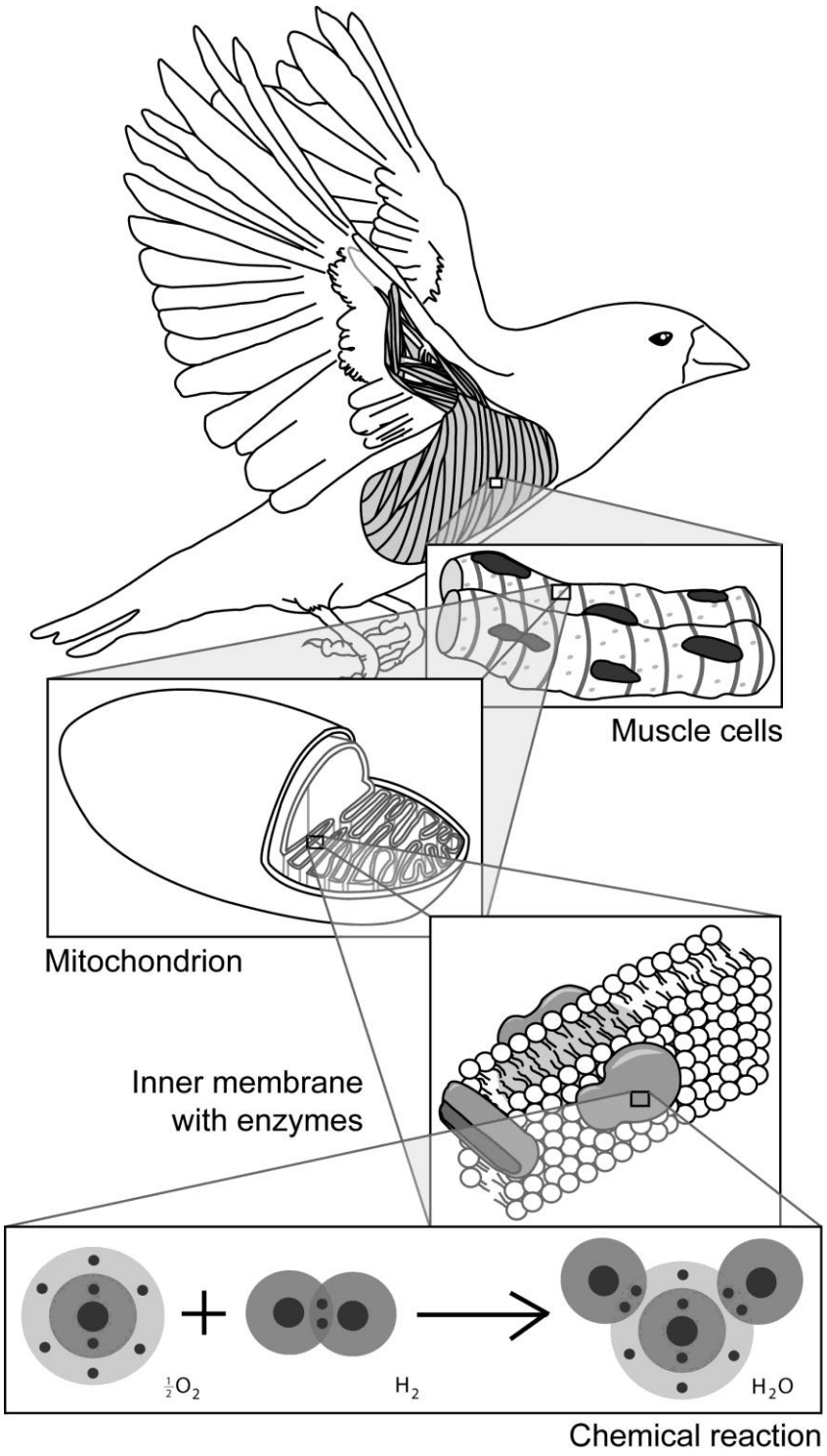

Fig. 1.1. Although the oxidative status of birds has important implications at the organismal and ecological levels, understanding how oxidative damage occurs begins at the atomic level. Chemical reactions taking place in the mitochondria generate pro-oxidants during aerobic respiration.

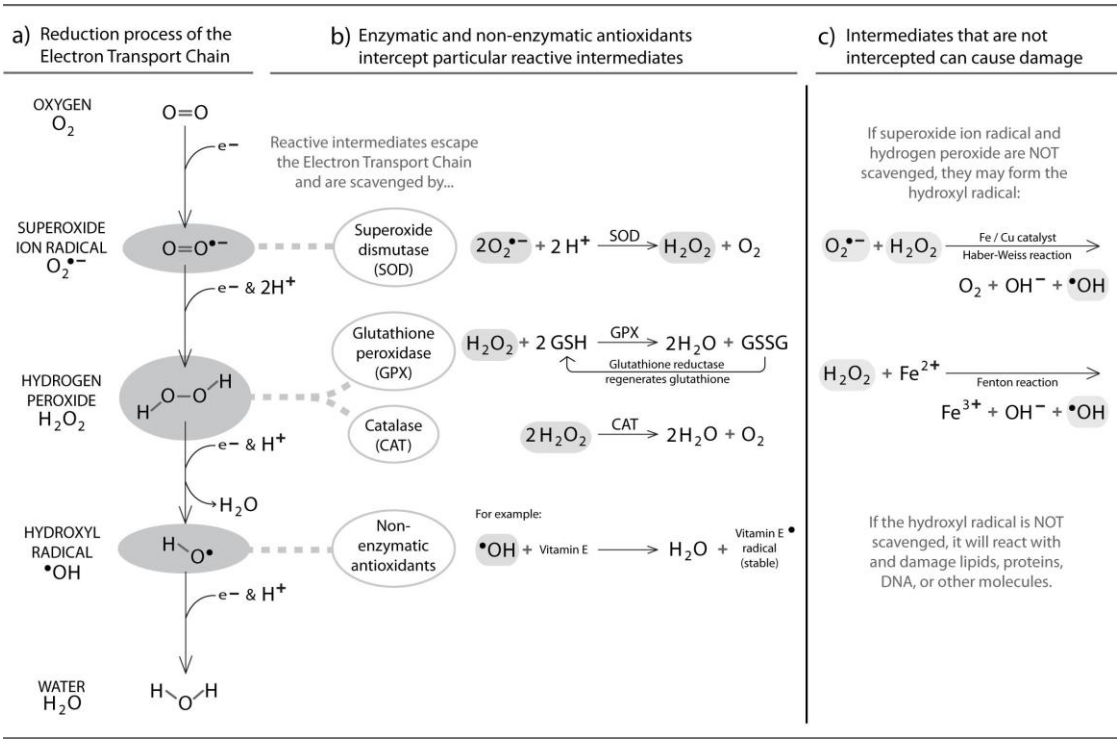

Fig. 1.2. The reduction of oxygen to water in mitochondria results in production of reactive intermediates, and layers of protection from by-products prevent oxidative damage.

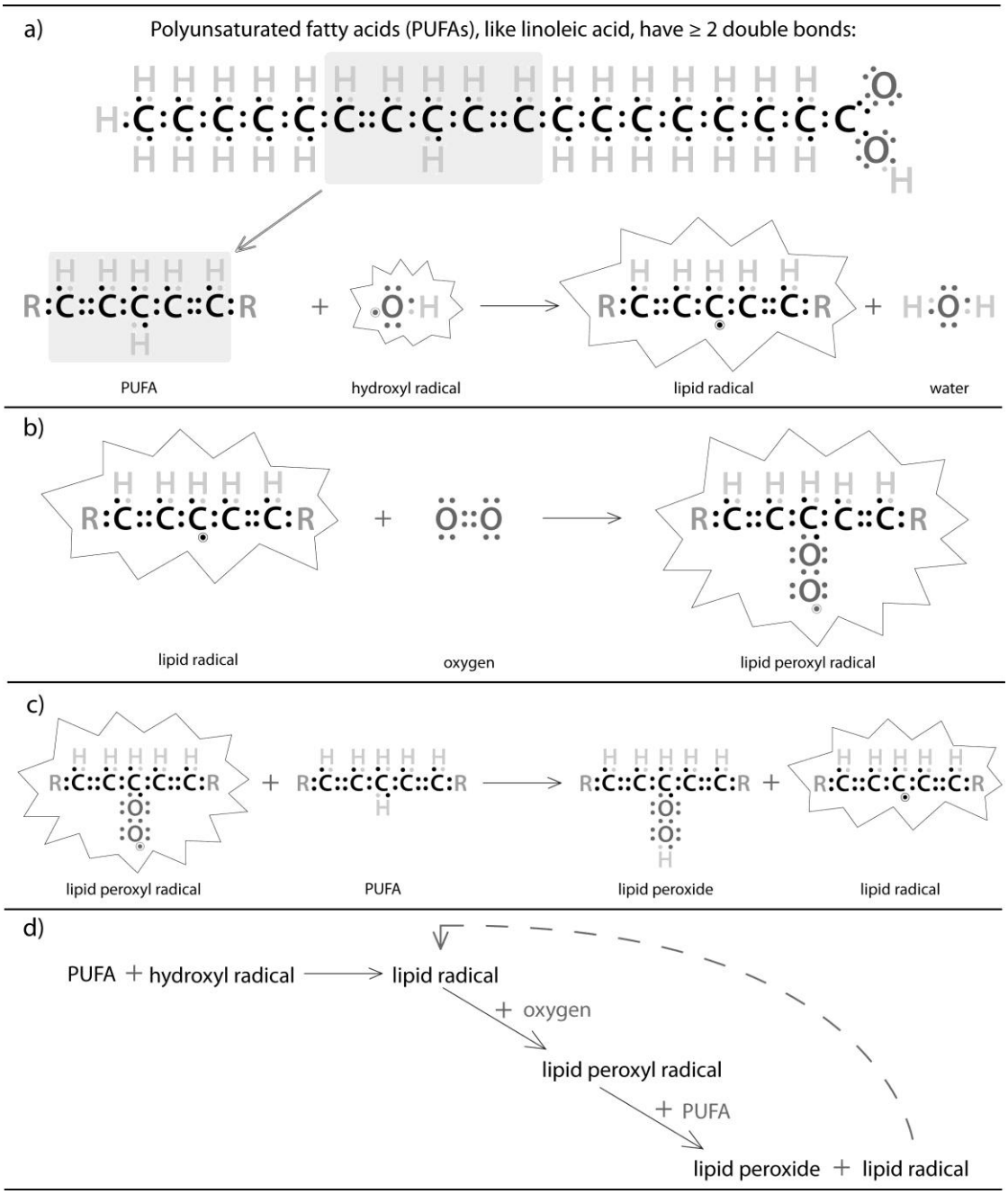

Fig. 1.3. Polyunsaturated fatty acids (PUFAs) such as linoleic acid are particularly vulnerable to oxidative damage. (a) A hydrogen atom near the double bonds is easily stolen by the hydroxyl radical (•OH), which has an unpaired electron, forming a lipid radical and water. (b) When the lipid radical encounters oxygen, it forms a lipid peroxyl radical. (c) The lipid peroxyl radical is then free to react with another intact PUFA, forming another lipid radical. (d) Overall, the entire process can be visualized as a chain reaction.

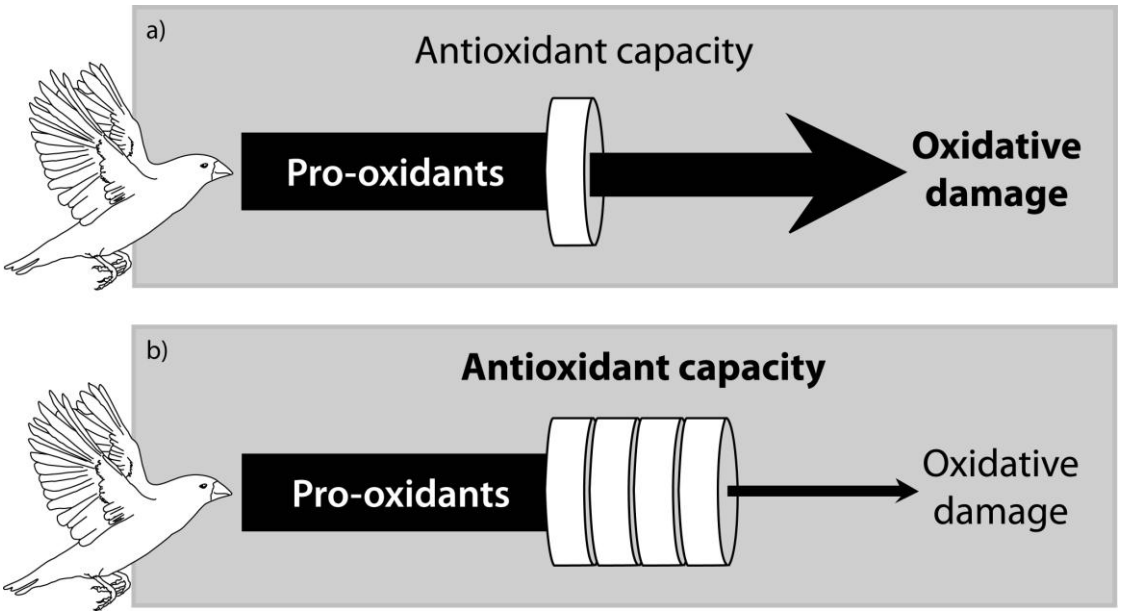

Fig. 1.4. The antioxidant capacity of an organism such as a flying bird can be visualized as a filter, intercepting pro-oxidants to prevent oxidative damage. Facing the same oxidative challenge, a bird (a) with little antioxidant capacity can be expected to suffer more oxidative damage than one (b) with greater antioxidant capacity (whether endogenous, dietary, or a combination of both). As described in the text and detailed in Table 1.1, birds can increase their antioxidant capacity in two ways: (1) up-regulate their endogenous antioxidant capacity (e.g., antioxidant enzymes), and (2) consume more dietary antioxidants in preparation for migration and during stopovers.

CHAPTER 2

Migrating songbirds on stopover prepare for, and recover from, oxidative challenges posed by long-distance flight

by

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Abstract

Managing oxidative stress is an important physiological function for all aerobic organisms, particularly during periods of prolonged high metabolic activity, such as long-distance migration across ecological barriers. However, no previous study has investigated the oxidative status of birds at different stages of migration and whether that oxidative status depends on the condition of the birds. In this study, we compared (1) energy stores and circulating oxidative status measures in (a) two species of Neotropical migrants with differing migration strategies that were sampled at an autumn stopover site before an ecological barrier, and (b) a species of trans-Saharan migrant sampled at a spring stopover site after crossing an ecological barrier; and (2) circulating oxidative measures and indicators of fat metabolism in a trans-Saharan migrant after stopovers of varying duration (0–8 nights), based on recapture records. We found fat stores to be positively correlated with circulating antioxidant capacity in Blackpoll Warblers and Red-eyed Vireos preparing for fall migration on Block Island, USA, but uncorrelated in Garden Warblers on the island of Ponza, Italy, after a spring crossing of the Sahara Desert and Mediterranean Sea. In all circumstances, fat stores were positively correlated with circulating lipid oxidation levels. Among Garden Warblers on the island of Ponza, fat anabolism increased with stopover duration while oxidative damage levels decreased. Our study provides evidence that birds build antioxidant capacity as they build fat stores at stopover sites before long flights, but does not support the idea that antioxidant stores remain elevated in birds with high fuel levels after an ecological barrier. Our results further suggest that lipid oxidation may be an inescapable hazard of using fats as the primary fuel for flight. Yet, we also

show that birds on stopover are capable of recovering from the oxidative damage they have accrued during migration, as lipid oxidation levels decrease with time on stopover. Thus, the physiological strategy of migrating songbirds may be to build prophylactic antioxidant capacity in concert with fuel stores at stopover sites before a long-distance flight, and then repair oxidative damage while refueling at stopover sites after long-distance flight.

Key-words antioxidant capacity, condition dependency, ecological barriers, lipid oxidation, long-distance flight, migration strategy

Introduction

Managing oxidative stress is an important physiological function for all aerobic organisms, particularly during periods of high metabolic activity like exercise, when mitochondria increase generation of harmful pro-oxidants (Niess 2005). Long-distance endurance exercise such as migratory flight poses an especially high oxidative challenge for bird species, particularly those that cross ecological barriers with little opportunity for rest or recovery. Migrating songbirds presumably respond to an increased potential for oxidative stress by up-regulating protective endogenous systems or accumulating dietary antioxidants at stopover sites (Costantini et al. 2007; Alan et al. 2013; Bolser et al. 2013; Jenni-Eiermann et al. 2014). Yet, no studies to date have investigated the oxidative status of birds at different stages of migration, at stopover sites before and after ecological barriers, and whether that oxidative status depends on the condition of the birds.

During migration, birds spend considerable time and energy at stopover sites, where they accumulate the necessary nutritional resources for completing the next leg of their journeys. At stopover sites, migratory songbirds build their fat stores in relation to the distance they must fly to reach their destination, and to meet energetic challenges (e.g., storms or barriers) they face along the way (Berthold 1993, 1996; Schaub and Jenni 2000; Fransson et al. 2008; Schaub, et al. 2008). Songbirds then use stopover sites after long flights to recover fat, lean, and/or water mass they have lost during the course of those flights (e.g., Leberg et al. 1996). Presumably, songbirds facing long flights or ecological barriers must also build up adequate protection, in the form of antioxidants, to prevent the oxidative damage possible from their own metabolism; that is, they should build and use antioxidant protection as they build and use their fat stores. Yet, studies of this phenomenon are lacking. Birds might also use stopover sites after long flights to replace exhausted antioxidants and/or purge oxidative damage. However, no studies have tracked changes in oxidative status in relation to stopover duration.

In this study, we examined the oxidative status of three species of songbirds, in relation to their fuel stores, under several migration scenarios and contexts: (1) during the early stage of fall migration when Blackpoll Warblers (*Setophaga striata* Forster 1772) and Red-eyed Vireos (*Vireo olivaceus* Linnaeus 1766) rest and refuel at an island stopover site off the coast of southern New England, USA, before they head south over the Atlantic Ocean for thousands of kilometers of sustained flight or travel along the western Atlantic coast; and (2) during the late stage of spring migration when Garden Warblers (*Sylvia borin* Boddaert 1783) rest and refuel at a stopover site

off the coast of Italy, immediately after flying hundreds of kilometers over the Mediterranean Sea from winter quarters in sub-Saharan Africa. We evaluated the following hypotheses and predictions: (Hypothesis 1) songbirds prepare for a migratory flight by building both their nonenzymatic antioxidant capacity and fat stores, as they likely consume foods rich both in antioxidants and fat, and therefore these measures will be correlated in birds captured prior to departure on a long migratory flight; (Hypothesis 2) birds use both their energy stores and antioxidant stores during migration, and therefore these measures will be correlated at a stopover site after an ecological barrier; (Hypothesis 3) birds with greater fat stores have a higher risk of oxidative damage, given that fat deposits are vulnerable to pro-oxidants generated during metabolism, and therefore circulating lipid peroxidation levels will correlate with fat stores in all circumstances; (Hypothesis 4) birds rebuild both their antioxidant capacity and fat stores on stopover after an ecological barrier, and therefore newly landed birds will have lower measures of both antioxidant capacity and fat anabolism than birds residing longer on stopover, and both measures will correlate with time on stopover.

Materials and methods

Field techniques – Block Island, RI, USA

We used mist nets to capture Blackpoll Warblers and Red-eyed Vireos on Block Island, Rhode Island, USA, between 5 September and 31 October 2012 and 2013. Block Island is a small glacially deposited island 15.5 km off the southern coast of Rhode Island (41º 13’ N, 71º 33’ W) and an important stopover site for millions of

migrating songbirds each autumn (Reinert et al. 2002). Blackpoll Warblers breed in the Canadian boreal forest and Alaska and then gather on the northern Atlantic coast of the United States in autumn for a trans-oceanic migration to South America (Baird 1999, Vuilleumier 2009, DeLuca et al. 2015). This species is therefore unique among the passerines that migrate along the western Atlantic coast, in that it accumulates considerable fat stores (doubling a lean mass of 9–11 g) in preparation for long-duration flights over the ocean to wintering grounds in northern South America, rather than take routes offering a number of coastal or inland stopover sites for refueling (Baird 1999). Unlike the Blackpoll Warbler, the Red-eyed Vireo travels over land to its wintering areas and accumulates less fat in autumn (ca. 5 g above lean mass; Cimprich et al. 2000); this species has the longest migration of the North American vireos, breeding in northeastern North America and traveling in autumn through Central America and the Caribbean to the Amazon basin (Harris 2009). Thus, Red-eyed Vireos stopping over on Block Island during fall are preparing for the next leg of their overland southbound journey like most Neotropical migrants, and may therefore be expected to have lower fat and antioxidant levels than Blackpoll Warblers.

Mist nets were operated on Block Island from 30 min before dawn until no later than 1600 local time. Within 1 h of a bird entering the mist net, we measured subcutaneous fat score on a 0–8 scale (Kaiser 1993) and body mass to 0.1 g. For 56 hatch-year Blackpoll Warblers and 63 hatch-year Red-eyed Vireos, we drew a ca. 150-µL blood sample to measure nonenzymatic plasma antioxidant capacity and plasma oxidative damage. All blood samples were centrifuged within 30 min of capture for 6

min at 5,000 rpm, and the plasma was flash frozen under liquid nitrogen. Plasma samples were later stored in the laboratory at –80°C until analysis.

The range of fat scores observed in Red-eyed Vireos was expected to be smaller than in Blackpoll Warblers, and so we also quantitatively measured fat mass of Red-eyed Vireos using the deuterium dilution method (Karasov and Pinshow 1998, McWilliams and Whitman 2013). After drawing a blood sample for plasma metabolites, we injected each bird with 50 µL of 99% deuterium-enriched water (D_2O) and drew a second 150-µL blood sample after a 60-min equilibration period in a cloth bag (see McWilliams and Whitman 2013 for details). These blood samples were flame-sealed and stored at 4°C until later analysis in the laboratory (described below). All work on Block Island was approved by the University of Rhode Island's Institutional Animal Care and Use Committee (IACUC # AN09-09-008).

Field techniques – Ponza, Italy

We used mist nets to capture Garden Warblers on Ponza, Italy, between 10 and 19 May 2012. Ponza is a small volcanic island 50 km off the Tyrrhenian coast of Italy (40º 55' N, 12º 58' E), where thousands of songbirds on broad-front northward migration stopover after flying long durations (≥10 h) from Africa in spring (Grattarola et al. 1999). The Garden Warbler breeds in west temperate Eurasia and winters in sub-Saharan Africa, crossing the Sahara Desert and Mediterranean Sea during fall and spring migrations; this species has been the subject of many migration studies (Berthold 1993; Grattarola et al. 1999; Biebach et al. 2000; Bauchinger et al.

2005), including those recently focused on stopover physiology on or near Ponza (Costantini et al. 2007; Fusani et al. 2009, 2011; Goymann et al. 2010).

Mist nets were operated on Ponza from 30 min before dawn until no later than 1 h after sunset. Within 1 h of capture, we measured subcutaneous fat score on a 0–8 scale (Kaiser 1993), size of the pectoral muscles scored on a 0–3 scale (Gosler 1991), and body mass to 0.1 g. For 129 newly captured individuals, we drew a ca. 150-µL blood sample within 3 min of a bird entering the mist net to measure the following parameters: nonenzymatic antioxidant capacity, oxidative damage, uric acid, β-hydroxybutyrate, triglycerides, and non-esterified fatty acids. All blood samples were centrifuged in the field for 6 min at 5,000 rpm, and the plasma was flash frozen under liquid nitrogen within 15 min of capture. Plasma samples were later stored in the laboratory at –80°C until analysis. For 45 of these 129 birds, we also measured fat mass using the deuterium dilution method, as described above. An additional 26 Garden Warblers were placed in cloth handling bags for between 20 and 162 min (mean = 95 min) before blood sampling, to evaluate changes in blood metabolites with time.

We stratified sampling to include Garden Warblers sampled on both high-capture and low-capture days. On Ponza and other islands in the Mediterranean Sea, high-capture days are characterized by large influxes of birds on broad-front migration, which makes it more likely that birds on these days just arrived to the island after a ≥ 10-h flight. We conservatively defined a high-capture day as one with > 1,000 total captures, > 90% of captures occurring after noon (marking the arrival of birds from the African coast), and capture rates after noon > 100 birds per hour. We

conservatively defined a low-capture day as one with equivalent netting effort but < 200 total captures, < 50% of captures occurring after noon, and capture rates after noon < 30 birds per hour.

We also drew blood samples for circulating metabolites in the manner described above for 24 individuals that had been previously captured during normal ringing operations on Ponza, and that were opportunistically recaptured between 1 and 192 h after initial capture. These individuals constituted a recaptures dataset with a known minimum duration of stopover. Two birds were blood-sampled twice, on two capture occasions, and therefore provided information on intra-individual trends in blood metabolites. All work on Ponza was performed under permission number A00785 as of 8 February 2013 from the government of the Regione Lazio according to Italian law.

Measurement of plasma metabolites

We used commercial kits modified for small volumes to perform metabolite assays for each of the 56 Blackpoll Warblers and 63 Red-eyed Vireos on Block Island, and the 129 newly captured and 24 recaptured Garden Warblers on Ponza. Sample sizes listed in the results are not equal across all assays because not enough plasma was available to complete all assays for each individual. We performed all metabolite assays at the University of Rhode Island using a microplate spectrophotometer (Biotek Powerwave 340, Winooski, VT). We ran samples in duplicate unless a coefficient of variation > 15% was observed, in which case we ran a third replicate if sufficient plasma was available. We determined antioxidant capacity as the ability of a plasma sample to

neutralize an oxidizing assault of hypochlorous acid, using the OXY-Adsorbent Test (concentration unit = mmol/L of HClO neutralized; Diacron International, Grosseto, Italy); and measured oxidative damage as the presence of circulating hydroperoxides, which include products of lipid oxidation, using the d-ROMs test (concentration unit = mmol/L H_2O_2 equivalents; Diacron International, Grosseto, Italy; see Costantini et al. 2007 for further details). We chose the OXY and d-ROMs tests as general whole-animal markers of circulating oxidative status, and to facilitate comparison of results with previous studies. We anticipated that OXY, as a measure of plasma nonenzymatic antioxidant capacity, would be particularly relevant in birds acquiring dietary antioxidants (Beaulieu and Schaefer 2014) on stopover as they consume fats; the d-ROMs test provides an index of damage to fats, and has been shown recently to covary with circulating triglyceride levels (Pérez-Rodriguez et al. 2015).

We used endpoint assays to measure uric acid (concentration unit = mmol/L; Teco Diagnostics Anaheim, CA), triglycerides (concentration unit = mg/mL; Sigma Aldrich Corporation, St. Louis, MO), and non-esterified fatty acids (concentration unit = mEq/L; Wako Diagnostics, Richmond, VA), and a kinetic assay to measure β-hydroxybutyrate (concentration unit = mmol/L; R-Biopharm, Darmstadt, Germany; Pierce et al. 2005; Smith et al. 2007; Smith and McWilliams 2009). Such metabolite assays are often used to track lipid uptake and deposition during feeding (circulating triglycerides), fat breakdown during exercise or fasting (β-hydroxybutyrate and non-esterified fatty acids), and protein catabolism (uric acid, which also functions as an endogenous antioxidant) in songbirds (Jenni-Eiermann et al. 2002; Pierce et al. 2005; Smith and McWilliams 2009, 2010).

Determination of fat mass via deuterium dilution

We estimated fat mass of 62 Red-eyed Vireos and 45 Garden Warblers injected with deuterium, given the volume of deuterium injected, the measured concentration of deuterium in plasma, and the predictive models and approach described in McWilliams & Whitman (2013). Briefly, we microdistilled the water from each flame-sealed blood sample and used infrared spectrophotometry to measure deuterium concentration (Karasov et al. 1988). Deuterium concentration was measured at the University of Rhode Island using a FT-IR Spectrometer with a Universal ATR Sampling Accessory, with Spectrum and Spectrum Quant software (PerkinElmer, Waltham, MA). We verified the mass of D_2O injected into birds (0.055 g) by weighing capillary tubes of the same injection volume, randomly selected from samples taken in the field. We then used this injection mass, the molar masses of D_2O (20 g/mol) and unlabeled H_2O (18 g/mol), and the deuterium enrichment of distilled blood water as determined by spectrophotometry to calculate estimated water space (Karasov and Pinshow 1998, McWilliams and Whitman 2013). We excluded from the dataset any values of estimated water space that were either <50% or >80% of body mass, given that these values are biologically unreasonable (McWilliams and Whitman 2013) and suggest that the samples were compromised. Fifteen Red-eyed Vireos and seven Garden Warblers were excluded from the dataset because their values were >80%, leaving 47 Red-eyed Vireo samples and 38 Garden Warbler samples for statistical analysis. We used the interspecific model in McWilliams and Whitman (2013) to estimate fat mass of Garden Warblers given estimated water space and measured body mass, and the model for Red-eyed Vireo in McWilliams and Whitman (2013) to

estimate fat mass of Red-eyed Vireos given estimated water space and measured body mass.

Statistical analyses

All statistical analyses were performed with SAS 9.4 software (SAS Institute 2014). We used nonparametric Spearman coefficients for all correlation analyses, given that variables of interest were not all normally distributed, even when transformed.

Newly captured Blackpoll Warblers, Red-eyed Vireos, and Garden Warblers

We used the CORR procedure to perform correlation analysis, to compare fat score, fat mass determined by deuterium dilution, and muscle score with antioxidant capacity and oxidative damage levels in newly captured birds, by species. We used the MIXED procedure to perform ANCOVAs (covariate = fat score), to compare antioxidant capacity and oxidative damage between Blackpoll Warblers and Red-eyed Vireos (treatment = species); we estimated variances separately for each species, given that the assumption of homogenous residual variance was violated. We therefore report effects tests with adjusted degrees of freedom, using the Kenward-Roger adjustment, to accommodate this more complicated covariance structure (Kenward and Roger 1997; Schaalje et al. 2002). For the 26 Garden Warblers held in cloth bags, we used correlation analysis to compare plasma metabolites and time before sampling.

Garden Warblers captured on high-capture vs. low-capture days

Although we captured 129 previously unbanded Garden Warblers, the amount of time that they had spent on the island before capture could not be determined with certainty for most individuals. However, if we assume that the probability of capturing a newly arrived bird is higher on a high-capture day, then a comparison of the 28 high-capture-day birds with the 19 low-capture-day birds may indicate how plasma metabolites change with time at a stopover site. We used t-tests to compare the following variables in birds caught on high-capture and low-capture days: antioxidant capacity, oxidative damage, uric acid, β-hydroxybutyrate (log-transformed to achieve a normal distribution), triglycerides (also log-transformed), and non-esterified fatty acids.

Recaptured Garden Warblers

We used data from Garden Warblers with known minimum durations of stay on the island (from recapture records) to assess the correlation between elapsed time and blood metabolites. Two birds were sampled twice, on two capture occasions. To ensure data independence, we used only the first measurements from these two individuals for the correlation analyses, but we report both measurements in the Results section.

Results

Newly captured Blackpoll Warblers, Red-eyed Vireos, and Garden Warblers

Block Island, USA

Fat score was positively correlated with both antioxidant capacity (n = 57) and oxidative damage (n = 33) among Blackpoll Warblers stopping over during fall migration on Block Island (Fig. 2.1). Among Red-eyed Vireos, fat mass was positively correlated with antioxidant capacity (n = 45) and oxidative damage (n = 30) (Fig. 2.1); fat score was also positively correlated with antioxidant capacity (n = 62, r_S = 0.357, P = 0.004) but not with oxidative damage (n = 40, r_S = 0.275, P = 0.086). Fat mass and fat score of Red-eyed Vireos were positively correlated (n = 47, r_S = 0.60, $P < 0.001$). In both species, antioxidant capacity and oxidative damage were not significantly correlated (Blackpoll Warbler n = 33, r_S = 0.295, P = 0.095; Red-eyed Vireo n = 41, r_S = 0.222, P = 0.162).

We performed ANCOVA (covariate = fat score) to compare antioxidant capacity between Blackpoll Warblers and Red-eyed Vireos (treatment = species); the fat score * species interaction was insignificant and therefore removed from the model ($F_{1,107}$ = 1.58; P = 0.21). Blackpoll Warblers (n = 57) had significantly lower antioxidant capacity (LS mean ± SE, 181.44 ± 4.88 mmol/L HClO neutralized) than Red-eyed Vireos (n = 62, 195.90 ± 3.31) (species $F_{1,98.4}$ = 5.94, P = 0.016; fat score $F_{1,102}$ = 12.53, P = 0.0006; Fig. 2.2). We performed ANCOVA to also compare oxidative damage between species; the fat score * species interaction was insignificant and therefore removed from the model ($F_{1,68.7}$ = 0.47; P = 0.50). Blackpoll Warblers (n = 33) had significantly higher circulating lipid peroxidation levels (48.82 ± 2.23 mmol/L H_2O_2 equivalents) than Red-eyed Vireos (n = 40, 31.10 ± 1.23) (species $F_{1,49.8}$ = 48.31, $P < 0.0001$; fat score $F_{1,65.2}$ = 20.17, $P < 0.0001$; Fig. 2.2).

Ponza, Italy

Fat score was not correlated with either antioxidant capacity (n = 123, r_S = 0.118, P = 0.195) or oxidative damage (n = 123, r_S = 0.118, P = 0.195) among migrating Garden Warblers newly captured on spring stopover (Fig. 2.3). Antioxidant capacity and oxidative damage were uncorrelated (n = 129, r_S = 0.067, P = 0.452). Fat mass was uncorrelated with antioxidant capacity (n = 37, r_S = 0.060, P = 0.726), but positively correlated with oxidative damage (n = 37, r_S = 0.485, P = 0.002) (Fig. 2.3). Muscle score was not correlated with either antioxidant capacity (n = 123, r_S = 0.090, P = 0.324) or with oxidative damage (n = 123, r_S = 0.164, P = 0.070) (Fig. 2.3). Fat mass and fat score were positively correlated (n = 38, r_S = 0.48 P = 0.002), and when we excluded three questionable points with a fat score of zero and ≥2.0 g of fat mass, the correlation improved (n = 35, r_S = 0.68, P < 0.001). However, excluding these points did not significantly improve the correlation between fat score and oxidative measures.

Among the 26 Garden Warblers held in cloth bags before blood sampling, there was no significant correlation between time and oxidative measures (antioxidant capacity and oxidative damage; n = 26, r_S < |0.25|, P > 0.3) or between time and most of the plasma metabolites (uric acid, triglycerides, non-esterified fatty acids; $n \geq 21$, r_S < |0.35|, P > 0.2). We did find that β-hydroxybutyrate increased with time before sampling (n = 23, r_S = 0.43, P = 0.039), clearly a consequence of forced fasting while in handling bags.

Garden Warblers captured on high-capture vs. low-capture days

Newly captured Garden Warblers sampled on high-capture days had lower plasma levels of triglycerides than low-capture-day birds (Fig. 2.4, Table 2.1). However, they did not significantly differ from birds sampled on low-capture days in their levels of non-esterified fatty acids, β-hydroxybutyrate, antioxidant capacity, oxidative damage, or uric acid. Fat scores of Garden Warblers sampled on high- and low-capture days were mostly 0–1 (94% of low-capture-day birds and 71% of high-capture-day birds).

Recaptured Garden Warblers

Antioxidant capacity and circulating uric acid of recaptured Garden Warblers did not change with minimum stopover time (n = 23, r_S = –0.018, P = 0.935; and n = 23, r_S = –0.185, P = 0.398, respectively) whereas oxidative damage significantly decreased with minimum stopover duration (n = 23; Fig. 2.5). All three measures of fat metabolism were significantly correlated with minimum stopover duration, with β-hydroxybutyrate and non-esterified fatty acid levels decreasing (n = 22 and 16, respectively) and triglyceride levels increasing (n = 20) the longer birds were on the island before blood sampling (Fig. 2.5).

Discussion

Our analyses provide the first assessment of oxidative status in songbirds with different migration strategies early in their migration before an ecological barrier, as well as evidence that birds recover from oxidative damage on stopover after long flights. Our study provides evidence that birds build antioxidant capacity as they build

fat stores at stopover sites before long flights, but does not support the idea that antioxidant stores remain elevated in birds with high fuel levels after an ecological barrier. We also show that birds on stopover are capable of recovering from the oxidative damage they have accrued during migration, as lipid oxidation levels decrease with time on stopover. Below we discuss these results as they relate to the four hypotheses tested, as well as the implications of our findings for understanding the effects of migration distance and body condition on the physiological strategies of songbirds.

Hypothesis 1: birds prepare for migration by building antioxidant capacity and fat stores

Costantini et al. (2007:369) suggested that birds in "good condition," that is, with high fuel stores, may have "reserves" of antioxidant capacity for controlling oxidative damage. Our study is the first to confirm this in free-living birds at a stopover site during migration. We found that antioxidant capacity and fat stores were positively correlated among both Blackpoll Warblers (fat score) and Red-eyed Vireos (fat mass) preparing for autumn migration at a northern stopover site, supporting the idea that birds build antioxidant capacity as they do energy stores before long-distance flights.

We expected Blackpoll Warblers to exhibit higher levels of antioxidant capacity than Red-eyed Vireos, given that the former would be preparing for an ocean crossing, but we did not find this to be the case. Rather, we found Blackpolls to have lower antioxidant capacity than vireos and significantly higher oxidative damage. This latter result would occur if the Blackpolls we captured had traveled a considerable

distance to reach Block Island; Blackpolls that summer in Canada may travel up to 2,500 km to reach staging areas in autumn on the east coast of the United States (Baird 1999), and evidence from several studies suggests that long flights increase oxidative damage (in pigeons, *Columba livia* Gmelin 1789, Costantini et al. 2008; in European Robins, *Erithacus rubecula* Linnaeus 1758, Jenni-Eiermann et al. 2014). The Red-eyed Vireos that we captured may have originated closer to Block Island than Blackpolls, and therefore sustained less oxidative damage before sampling.

Hypothesis 2: birds use both antioxidant capacity and fat stores during migration

In contrast to Costantini et al.'s (2007) findings for Garden Warblers on Ponza in spring, we did not find circulating nonenzymatic antioxidant capacity to be correlated with fat score, fat mass, or muscle score in this study. Costantini et al. (2007) hypothesized that individuals with higher energy stores before long flights probably also have higher initial circulating nonenzymatic antioxidant levels, and therefore are better able—during the course of those flights—to prevent oxidative stress than leaner/antioxidant-poorer individuals, even when all flew the same distance. However, we have no evidence to support their suggestion that birds with higher energy stores after migration face lower oxidative stress by maintaining higher antioxidant reserves. Actually, the antioxidant capacity values in our study were considerably lower than what Costantini et al. (2007) documented and what we found in Blackpoll Warblers and Red-eyed Vireos; the oxidative damage values of Garden Warblers in our study were within the range of what we found for Block Island birds but higher than those reported by Costantini et al. (2007).

There are three possible explanations for why our results for Garden Warblers on Ponza differed from those of Costantini et al. (2007). First, the smaller sample size (<30 individuals per species) in the 2007 study may have not captured the variation seen in the current study with 129 Garden Warblers. Second, birds that we captured may have flown a longer distance before reaching the island, or encountered fewer antioxidant-rich foods than the Garden Warblers sampled by Costantini et al. (2007). Third, our sample was collected 10–20 days later in May when the pace of migration may be more intense with the approaching breeding season. For example, Wojciechowski et al. (2014) suggested that the nutritional priorities of Blackcaps (*Sylvia atricapilla* Linnaeus 1758) on stopover may change depending on season and the urgency of reaching the breeding grounds. Recent work with Hermit Thrushes (*Catharus guttatus* Pallas 1811) on Block Island (Smith and McWilliams 2014) has shown that the relationship between condition and stopover behavior changes as the fall migration season progresses. We suggest that Garden Warblers captured in mid-to-late May could present higher oxidative damage levels and less condition dependency in antioxidant stores than birds captured earlier in spring migration. Furthermore, the antioxidant status of birds, and the availability of dietary antioxidants, may vary considerably between fall and spring, given that the pace of migration is generally faster in anticipation of breeding, and the phenology of most temperate plants dictates that the peak abundance of antioxidant-rich foods is in autumn. This leads us to hypothesize that although birds may build antioxidant capacity as they do fuel stores on stopover, the accumulation or use of these

antioxidants may depend not only on the duration of active flight but also on the pace of migration, including the length of stopover.

Hypothesis 3: extent of circulating lipid oxidative damage is related to amount of lipids stored

We confirmed in all three species we examined that fat stores in birds at stopover sites were positively correlated with lipid oxidative damage. Stored fats are vulnerable to attack by pro-oxidants when protective mechanisms increase only linearly with fat mass; therefore, a high amount of stored fat can be expected to produce a high amount of lipid oxidation products (Pérez-Rodriguez et al. 2015). Jenni-Eiermann et al. (2014) found that oxidative damage to proteins (measured as protein carbonyls) did not correlate with fat, and so lipid stores or damage to those stores may not reflect the levels of oxidation products from other tissue precursors (e.g., flight muscle). We observed considerable variation in the range of damage experienced by the three species, with Red-eyed Vireos showing the least variability in damage levels; this pattern might be explained by a greater diversity of population origins of the longer-distance Garden and Blackpoll Warblers at our study sites, although this explanation is only speculative and warrants further study with a greater suite of species and sites.

Many techniques are available for assessing oxidative status in animals (Costantini 2008; Monaghan et al. 2009), and controversy exists as to which measures, or combination of measures, may best represent an animal's overall condition or oxidative state (Costantini and Verhulst 2009; Cohen et al. 2010; Hõrak and Cohen 2010). Lipids are particularly important molecules for migratory birds, and other

animals that build large fat stores, for example, for migration or hibernation; and therefore lipid oxidation products may continue to be a highly relevant measure of oxidative damage in bird studies. However, we urge future researchers to consider, when measuring circulating levels of lipid hydroperoxides, the importance of accounting for total mass or volume of fat in an animal as a whole.

Hypothesis 4: birds recover both antioxidant and fat stores on stopover after a barrier

We collected two types of data to address how stopover duration after an ecological barrier affects migrating birds' oxidative status and fat metabolism. Both yielded the same conclusions: Garden Warblers switched from fat catabolism to anabolism to recover their fat stores during stopover on Ponza, and lipid oxidative damage decreased, but circulating antioxidant capacity did not change with stopover length. Our limited repeated-measures data from two individuals sampled twice on stopover further confirmed these trends. Remarkably, oxidative damage levels plummeted in these two individuals within several days of stopping over, and interindividual data showed decreasing hydroperoxide levels over time, presumably as birds increased fat anabolism and replaced damaged lipids.

Interestingly, uric acid levels and antioxidant capacity were not associated with length of stay on Ponza, or with the differentiation between high-capture-day and low-capture-day birds. The absence of a trend in uric acid likely indicates that birds did not increase their intake of dietary protein on stopover, given that uric acid is the final product of protein catabolism in birds and correlates closely with protein consumption

(Alan & McWilliams 2013). Alternatively, uric acid levels may have been similar between newly arrived birds and birds on stopover because the former catabolized body protein during flight and the latter catabolized dietary protein upon landing. The absence of a trend in antioxidant capacity with time on the island likely reflects the dearth of antioxidant-rich food resources in spring on Ponza. On Block Island and other fruit-rich stopover sites used in autumn, we suspect that birds increase their circulating antioxidant capacity as they consume antioxidant-rich fruits while stopping over, given that the autumn fruits birds consume most on Block Island tend to have the highest antioxidant content (Alan et al. 2013, Bolser et al. 2013). However, on Ponza and other fruit-poor stopover sites in spring, dietary antioxidants may be difficult to acquire.

Conclusion

Our study is the first to show that songbirds build circulating antioxidant capacity as they do fat stores while preparing for migration, and that flight distance before a stopover site may affect lipid oxidation levels. It does not, however, support the idea that antioxidant reserves remain in birds with high fuel stores after an ecological barrier. We further show that oxidative damage may be an inescapable hazard of using fats as the primary fuel for flight, but also that birds on stopover are capable of recovering from the oxidative damage they have accrued during migratory flight, as lipid peroxidation levels decrease with time on stopover. We further hypothesize that building circulating nonenzymatic antioxidant capacity on stopover likely depends on the resources available at the stopover site. Thus, the physiological strategy of

migrating songbirds may be to build prophylactic antioxidant capacity in concert with fuel stores at stopover sites before a long-distance flight, and then repair oxidative damage while refueling at stopover sites after long-distance flight. The costs and benefits of long-jump versus short-hop migration strategies have been mainly derived from optimality models concerned with saving time, saving energy, or a combination of both (e.g., Hedenström and Alerstam 1997). We suggest that, in future, the ability of birds to prevent or repair oxidative damage resulting from sustained flight periods should also be considered in the context of migration ecology and evolution.

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Data Accessibility

Data are available from the Dryad Digital Repository:

http://dx.doi.org/10.5061/dryad.sj6n2

Conflict of Interest

None declared.

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Table 2.1. Comparison of plasma measures of oxidative state and fat metabolism between Garden Warblers captured on high-capture or low-capture days. High-capture days are characterized by large influxes of birds on broad front migration, which makes it more likely that birds on these days had just arrived to the island after a ≥ 10-h flight.

N High-capture	Low-capture	Type of assay	Plasma measure	DF	*t*-value	*P*-value
28	19	Oxidative state	Antioxidant capacity[a]	45	–0.17	0.869
28	19	Oxidative state	Oxidative damage[b]	37.3	–1.32	0.193
28	19	Oxidative state	Uric acid[a]	45	–0.73	0.471
28	19	Fat metabolite	log β-hydroxybutyrate [a]	45	–1.82	0.076
25	18	Fat metabolite	log triglycerides[b]	30.4	5.08	< 0.0001
23	17	Fat metabolite	Non-esterified fatty acids[a]	38	–1.55	0.129

[a] variance calculation used = pooled
[b] variance calculation used = Satterthwaite.

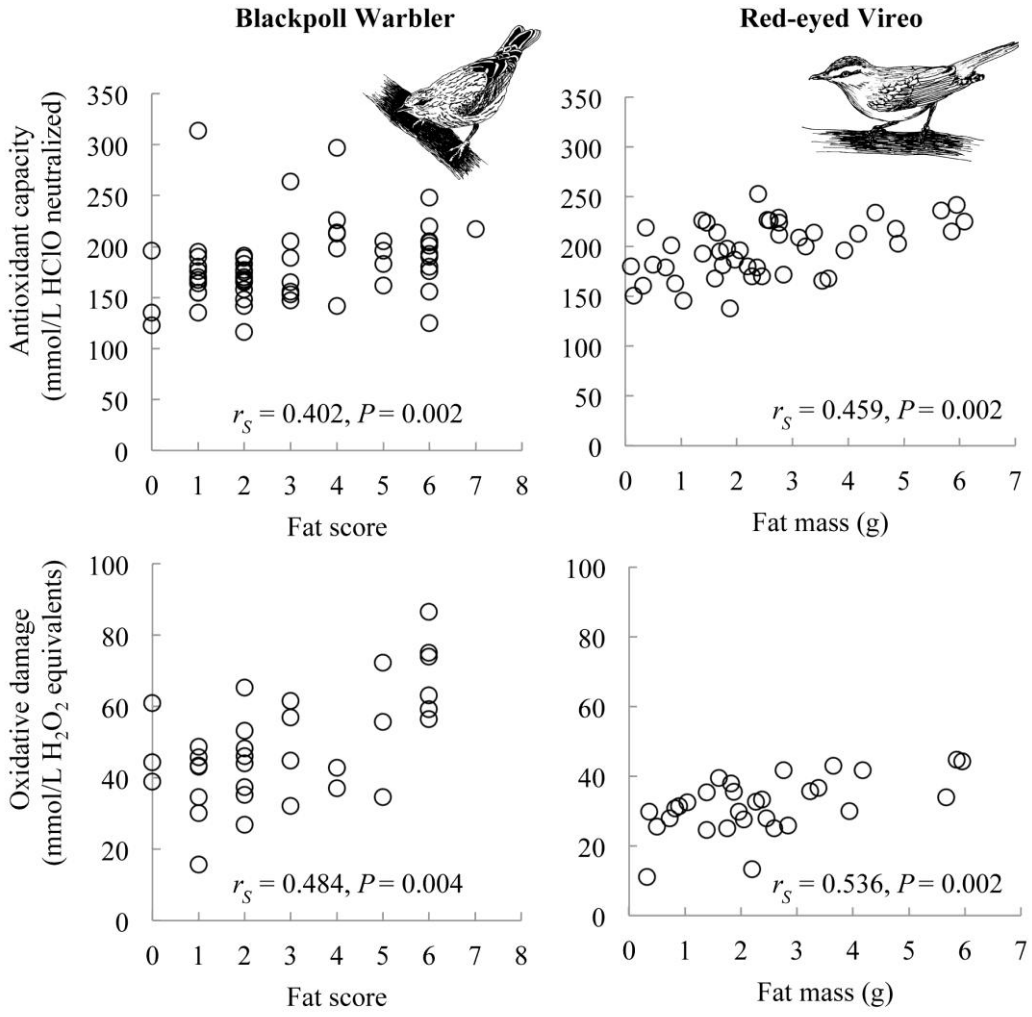

Fig. 2.1. Fat stores were correlated with both antioxidant capacity and oxidative damage among Blackpoll Warblers (left) and Red-eyed Vireos (right) in autumn on Block Island, USA. Although fat score was measured in Red-eyed Vireos, it was less strongly correlated with oxidative measures than fat mass.

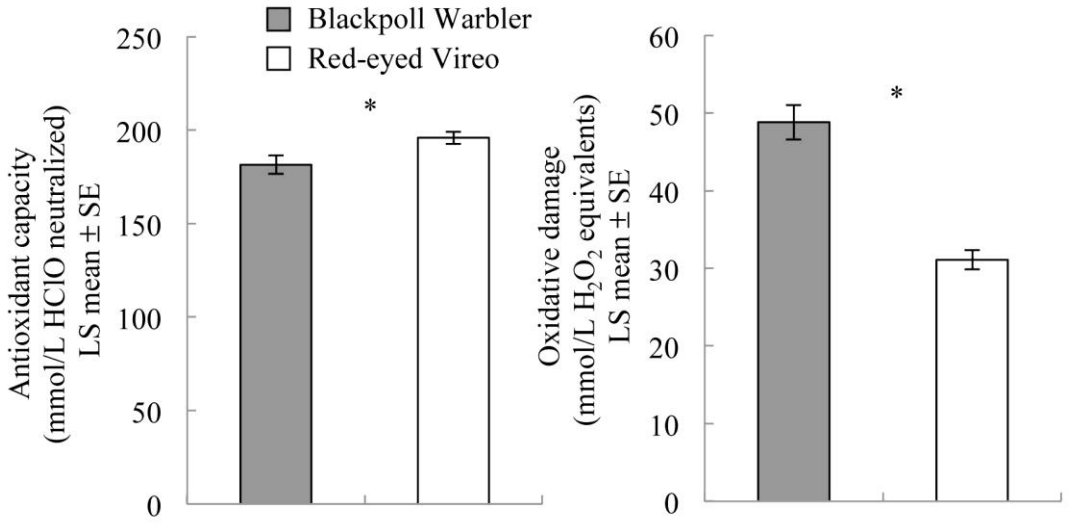

Fig. 2.2. Antioxidant capacity (ANCOVA, with fat score as covariate) was lower in Blackpoll Warblers than Red-eyed Vireos, and oxidative damage was higher in Blackpoll Warblers than Red-eyed Vireos in autumn on Block Island, USA. Asterisks indicate a significant difference between species ($P < 0.05$).

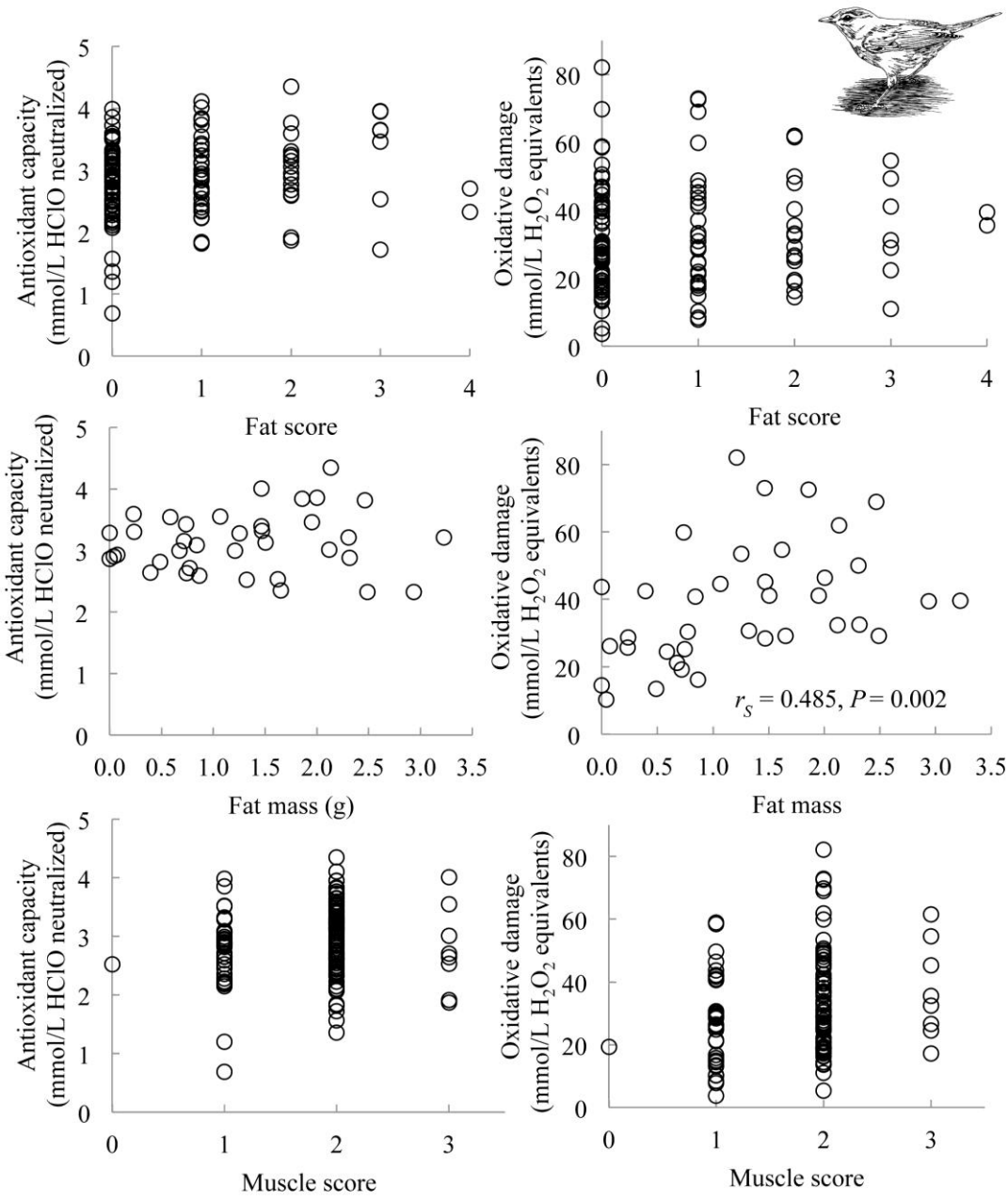

Fig. 2.3. Only fat mass was correlated with only oxidative damage among newly captured Garden Warblers in spring on Ponza, Italy; fat score and muscle score were uncorrelated with oxidative measures.

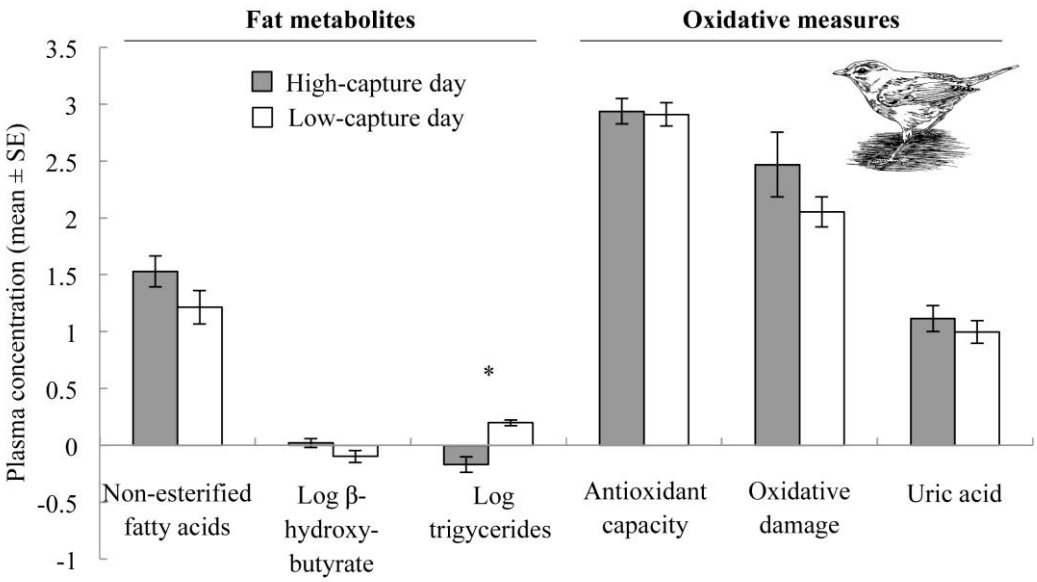

Fig. 2.4. Newly captured Garden Warblers sampled on high-capture days (filled bars) had similar oxidative measures to birds sampled on low-capture days (open bars), but dissimilar plasma fat metabolites, particularly triglycerides. See Table 2.1 for t-statistics and *P*-values, and Methods for concentration units for each plasma measure. Oxidative damage values displayed here are divided by 10 to fit on the same axis as other measures.

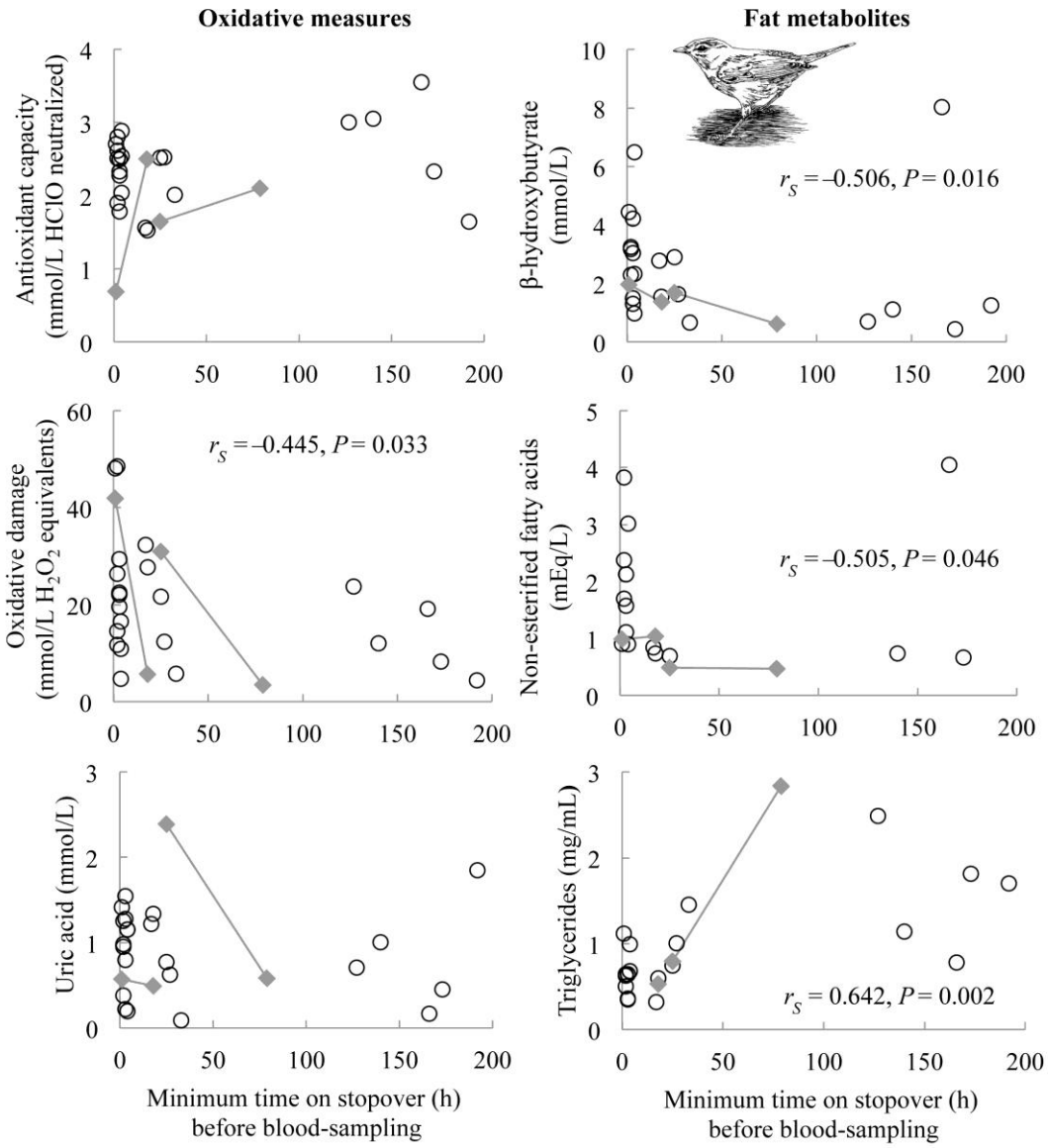

Fig. 2.5. Levels of six plasma blood metabolites from migrating Garden Warblers recaptured during stopover on Ponza in relation to stopover duration (correlation statistics shown only when $P < 0.05$). Minimal time on stopover (h) is the duration between recapture records. Intra-individual time-series data were available from two individuals that were blood-sampled twice (filled diamonds with connected lines).

CHAPTER 3

Access to water affects the condition dependency of nocturnal restlessness in Garden Warblers on a Mediterranean island stopover

by

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ABSTRACT

During migration, many songbirds encounter large ecological barriers, like deserts and seas that require substantial fuel to cross and can lead to dehydration during passage. If muscle is not catabolized to generate metabolic water, birds must seek free water on a subsequent stopover to replenish the water lost. Yet, no work has examined if birds crossing large migration barriers use access to free water in concert with energy or protein stores to make stopover decisions. We captured 61 free-living Garden Warblers (*Sylvia borin*) in spring at a frequently used stopover site in the Mediterranean Sea, housed them with or without drinking water, and measured nocturnal restlessness (Zugunruhe) in relation to energy stores at capture. Both groups lost equivalent flight muscle mass overnight, suggesting that water-deprived birds did not preferentially catabolize this tissue to produce metabolic water. Fat score and body mass, but not flight muscle mass, were positively correlated with nocturnal activity in both treatment groups. However, the slope of the relationship between nocturnal activity and fat score differed between groups, with water-deprived birds of high fat score showing the highest Zugunruhe activity. Our results suggest that birds with large energy stores use access to drinking water to inform their decisions about when to depart from a stopover site. Individuals with higher fat scores might be expected, regardless of flight muscle size, to depart a dry stopover site more readily than a site with freely available water. We suggest follow-up studies to further elucidate the mechanisms of this phenomenon and establish its prevalence in free-living birds.

KEYWORDS

condition dependency; dehydration; migration; stopover; Zugunruhe

INTRODUCTION

During migration, many songbirds encounter large ecological barriers, such as deserts and seas, which provide little or no opportunity to feed and drink. Crossing these barriers requires substantial energy, but can also result in dehydration when birds lose water due to evaporation during passage (Fogden 1972; Carmi et al. 1992; Klaassen 1996, 2004; Leberg et al. 1996). Birds replenish the fuel they have catabolized by feeding extensively on stopover. While energy stores and food resources are known to affect stopover duration and the birds' motivation to depart stopover sites (e.g., Fusani et al. 2009, 2011; Goymann et al. 2010; Eikenaar and Bairlein 2014; Smith and McWilliams 2014), the effect of water availability on the birds' stopover behavior has received little attention (Sapir et al. 2004; Tsurim et al. 2008; Mizrahy et al. 2011). Particularly on remote islands in the Mediterranean Sea, or other locations receiving extensive landfall of songbirds after barrier crossings, dehydration may influence how body condition determines the timing of the onward migration.

Recent studies of spring-migrating Garden Warblers (*Sylvia borin*) on the islands of Ponza and Ventotene, Italy, have shown that body condition is related to intensity of nocturnal migratory restlessness (Zugunruhe) in caged birds and stopover duration in free-ranging birds outfitted with transmitters (Fusani et al. 2009, 2011; Goymann et al. 2010). These studies used visible fat and pectoral muscle size as an

index of condition, but did not consider how water availability may affect condition dependency. Ponza is a particularly interesting study site for investigating the effects of water availability, given that thousands of songbirds stopover on the island after flying long durations (> 10 h) from Africa in spring (Grattarola et al. 1999), and free water on the island is not abundant.

In the experiment presented here, we examined how songbirds respond to water deprivation after crossing a large ecological barrier, whether by preferentially catabolizing flight muscle to replenish body water, or attempting to leave the island due to increasing nocturnal restlessness (Zugunruhe), which has been recently validated as a reliable proxy for departure likelihood (Eikenaar et al. 2014). We captured 61 free-living Garden Warblers on Ponza during spring migration, housed them for 24 h with or without drinking water, and measured Zugunruhe in relation to energy stores at capture. We also used the deuterium dilution method of fat mass estimation (Karasov and Pinshow 1998; McWilliams and Whitman 2013) to measure lipid stores, given that birds with low fat scores may still have visceral fat in significant amounts (Maillet and Weber 2006) and vary considerably in total fat content (McWilliams and Whitman 2013). Past studies (e.g., Fusani et al. 2009, 2011) that found relatively modest correlations between intensity of Zugunruhe and fat score may have benefited from using the deuterium dilution method, which estimates both readily visible and visceral adipose tissue, but the effect of dehydration on this method has not been fully evaluated. Furthermore, we evaluated two intervals of Zugunruhe behavior: (a) the early night period when birds are most likely to depart from a stopover site (e.g., Biebach et al. 2000; Goymann et al. 2010) and, therefore, when

condition dependency might be strongest; and (b) all night, when has been traditionally evaluated in previous experiments (e.g., Fusani et al. 2009, 2011).

Our objectives were to (1) determine how access to water affects Zugunruhe activity and overnight changes in flight muscle size, and (2) relate whole-animal fat content estimated using deuterium dilution to visible fat score under conditions of water availability and water deprivation.

MATERIALS AND METHODS

Field techniques

We used mist nets to capture 61 Garden Warblers on the island of Ponza, Italy, between 10 and 13 May 2012. Ponza is a small volcanic island 50 km off the Tyrrhenian coast of Italy (40º 55' N, 12º 58' E). Within 1 h of capture, we measured subcutaneous fat scored on a 0–8 scale (Kaiser 1993), size of the pectoral muscles scored on a 0–3 scale (Gosler 1991), body mass to 0.1 g, and tarsus length to 0.01 mm. We also measured flight muscle shape at capture to 0.01 mm with a muscle meter (Bauchinger et al. 2011) and used muscle shape, body mass, and tarsus length to calculate total flight muscle mass, using the equation for Garden Warblers developed by Bauchinger et al. (2011): flight muscle mass = –1.212 + (0.293 x muscle shape) + (0.045 x body mass) + (0.199 x tarsus length).

Birds were housed individually overnight in 50 x 25 x 30 cm cloth cages so that they were visually isolated from each other. All birds were housed in an indoor

room accommodating 20 cages, with natural illumination provided through a large door. No food was provided to reduce the potential for confounding effects of food availability on Zugunruhe (Fusani et al. 2011; Eikenaar and Bairlein 2014). Individuals captured on 10 and 11 May 2012 (n = 12 and 17, respectively; total = 29) were housed without water or a water dish, whereas birds captured on 12 and 13 May (n = 15 and 17, respectively; 32 total) were provided ad libitum water in 11 x 7.5 x 3.5 cm dishes at the time of caging (by 1300 hrs). Zugunruhe (migratory restlessness) was measured overnight using an infrared activity sensor connected with an activity recorder on each cage. We counted the number of times the infrared sensor was activated for each 2-min period, and then calculated the average activity during two intervals: civil sundown to midnight and midnight to civil sunrise (GMT + 1) (Fusani et al. 2009, 2011). We used the first interval to represent "early-night" Zugunruhe and averaged the two intervals to represent "all-night" Zugunruhe. In the morning, fat score, body mass, and flight muscle shape were measured again, total flight muscle mass was calculated again as above, and we assessed fat mass using the deuterium dilution method: we injected 50 µL of 99% deuterium-enriched water (D_2O) into the breast musculature of each bird and drew a 150-µL blood sample after a 60-min equilibration period in a cloth bag [see McWilliams and Whitman (2013) for details]. Birds were subsequently released, and blood samples were flame-sealed and stored at 4°C until analysis. All work on Ponza was performed under permission number A00785 as of 8/2/2013 from the government of the Regione Lazio according to Italian law.

Determination of fat mass via deuterium dilution

We estimated fat mass of Garden Warblers injected with deuterium given the volume of deuterium injected, the measured concentration of deuterium in plasma, and the predictive models and approach described in McWilliams and Whitman (2013). Briefly, we microdistilled the water from each flame-sealed blood sample and used infrared spectrophotometry to measure deuterium concentration (Karasov et al. 1988). Deuterium concentration was measured at the University of Rhode Island using a FT-IR Spectrometer with a Universal ATR Sampling Accessory, with Spectrum and Spectrum Quant software (PerkinElmer, Waltham, MA, USA). We verified the mass of D_2O injected into birds (0.055 g) by weighing capillary tubes of the same injection volume, randomly selected from samples taken in the field. We then used this injection mass, the molar masses of D_2O (20 g/mol) and unlabeled H_2O (18 g/mol), and the deuterium enrichment of microdistilled blood water as determined by spectrophotometry to calculate estimated water space (Karasov and Pinshow 1998; McWilliams and Whitman 2013). We excluded from the dataset any values of estimated water space that were either <50% or >80% of body mass, given that these values are biologically unreasonable (McWilliams and Whitman 2013). In total, we excluded 14 samples; 10 of 11 exclusions among non-watered birds, and all 3 exclusions among watered birds, were > 80% of body mass. We used the interspecific predictive model presented in McWilliams and Whitman (2013) to estimate fat mass (g) given estimated water space and measured body mass.

Statistical analyses

All statistical analyses were performed with SAS 9.4 software (SAS Institute 2014). We set statistical significance at $\alpha = 0.05$.

We used correlation analysis to compare estimated fat mass based on the deuterium dilution method with fat scores, both recorded on the morning after all-night captivity, to determine if deprivation of drinking water for ≥12 h affected the utility of the deuterium dilution method.

To determine if Garden Warblers that were not provided with water preferentially catabolized protein overnight to release metabolic water, we performed repeated-measures (RM) ANOVA, comparing flight muscle mass pre- and post-overnight captivity (variable = Time), and tested the interaction between water treatment (Water) and time (Water x Time). We also performed RMANOVA with fat score and body mass pre- and post-overnight captivity. We tested residuals for normality and homogenous variance, to ensure satisfaction of model assumptions.

We also used correlation analysis, after Fusani et al. (2009, 2011), to assess the relationship between Zugunruhe and several indicators of body condition (fat score, body mass, flight muscle mass, and muscle score), separately for birds that were or were not provided with water, for both the "early-night" Zugunruhe period and for "all-night." We used non-parametric Spearman coefficients for all correlation analyses, given that variables of interest were not all normally distributed, even when transformed. In addition, we used ANCOVA to test the contribution of water treatment (categorical variable), condition parameters (covariate = fat score or body

mass), and their interaction (Water x Covariate) to "early-night" Zugunruhe behavior. The interaction test indicated whether the slope of the relationship between Zugunruhe and the condition differed with water treatment. We tested residuals for normality and homogenous variance, to ensure satisfaction of model assumptions.

RESULTS

Evaluation of the deuterium dilution method for estimating fat mass

Among Garden Warblers that were provided with water throughout the experiment, fat mass estimated by the deuterium dilution method was strongly correlated with the visible fat score the morning after all-night captivity ($n = 29$, $r_S = 0.73$, $p < 0.0001$), whereas a positive although not significant correlation existed among birds that were not provided with water ($n = 17$, $r_S = 0.45$, $p = 0.073$; Fig. 3.1). Furthermore, the deuterium dilution method resulted in low deuterium concentrations as measured in microdistilled blood water, and, consequently, there were biased-high water space estimates and biased-low fat mass estimates in ten of 29 water-deprived birds as opposed to only three of 32 birds that were provided water. The difference in number of exclusions between treatment groups indicates that non-watered birds were dehydrated whereas watered birds drank the water provided and so were normally hydrated. The lack of significant correlation for non-watered birds is likely related to these many exclusions along with, by chance, several fewer fat birds assigned to the water-deprived group.

Overnight changes in flight muscle, fat score, and body mass

Flight muscle mass decreased overnight for all birds, regardless of water treatment (RMANOVA; Time $F_{1, 59} = 134.82$, $p < 0.0001$; Water $F_{1, 59} = 1.41$, $p = 0.240$; Water x Time $F_{1, 59} = 0.11$, $p = 0.743$; Fig. 3.2). Similarly, overall body mass and fat score decreased overnight for all birds, regardless of water treatment (RMANOVA; body mass, Time $F_{1, 59} = 611.37$, $p < 0.0001$, Water $F_{1, 59} = 0.39$, $p = 0.537$, Water x Time $F_{1, 59} = 0.07$, $p = 0.789$; fat score, Time $F_{1, 59} = 161.44$, $p < 0.0001$, Water $F_{1, 59} = 2.33$, $p = 0.133$, Water x Time $F_{1, 59} = 0.65$, $p = 0.422$).

Condition dependency of early-night Zugunruhe (civil sundown to midnight)

During the early night, fat score and body mass at capture were strongly and positively correlated with Zugunruhe among all birds, whether or not they were provided with water, while flight muscle mass was uncorrelated, and muscle score was only weakly correlated with Zugunruhe in both groups (Table 3.1). Non-watered birds with fat scores of 5 at capture displayed higher Zugunruhe activities than all birds that were provided with water (Fig. 3.3), despite having average levels of flight muscle mass at capture (Fig. 3.4). ANCOVA confirmed this condition dependency of Zugunruhe, given that the fat score covariate was statistically significant and interacted with the water treatment (ANCOVA; Water $F_{1, 57} = 1.33$, $p = 0.25$; Fat score $F_{1, 57} = 23.71$, $p < 0.0001$; Water x Fat score $F_{1, 57} = 4.29$, $p = 0.04$). Therefore, the slope of the relationship between fat score and Zugunruhe differed between watered and non-

watered birds. ANCOVA also confirmed that body mass at capture correlated with Zugunruhe, but the body mass covariate did not significantly interact with the water treatment (ANCOVA; Water $F_{1, 57} = 2.95$, $p = 0.09$; Body mass $F_{1, 57} = 9.02$, $p = 0.004$; Water x Body mass $F_{1, 57} = 3.30$, $p = 0.07$).

Condition dependency of all-night Zugunruhe (civil sundown to civil sunrise)

Body mass and fat score at capture were less strongly correlated with all-night Zugunruhe activity than they were with early-night activity (Table 3.1). Fat score no longer correlated with activity for birds that were not provided with water, and body mass no longer correlated with activity for birds that were provided with water. As during early-night Zugunruhe, flight muscle mass was uncorrelated with activity in both groups, and muscle score was only weakly correlated.

DISCUSSION

Access to water affected condition dependency of Zugunruhe

Evidence from our experiment with Garden Warblers suggests that water availability during stopover affects the condition dependency of Zugunruhe, suggesting that free water is important to the behavioral choices of birds after sustained flights. First, we found that the slope of the relationship between fat stores and migratory restlessness differed when birds were or were not provided with drinking water. Previous work by

Fusani et al. (2009, 2011) on Ponza showed that energy stores affect migratory restlessness in Garden Warblers and other passerine migrants stopping over on the island. Our study adds that birds having just crossed the Mediterranean Sea are perhaps even more likely to leave the island with large energy stores when they do not encounter free drinking water. Specifically, water-deprived birds with high fat scores in our experiment had higher levels of early-night Zugunruhe than birds with a similar fat score but which had access to drinking water. Second, in a study conducted exclusively with birds provided water, Fusani et al. (2009) found that fat score at capture was the variable most closely correlated with all-night Zugunruhe in Garden Warblers stopping over on Ponza. Interestingly, the relationship was significant in our study only when birds were provided with water (Table 3.1). Indeed, we found the condition dependency of Zugunruhe to be strongest overall when considered during the early-night period, unsurprising given that birds are most likely to depart a stopover site shortly after sunset (Biebach et al. 2000; Goymann et al. 2010). In short, if an animal cannot find the resource(s) necessary to increase its body condition, it must continue migration as soon as possible (Biebach 1985; Gwinner et al. 1988; Newton 2008).

The "water hypothesis" posits that birds during migratory flight without access to free water catabolize protein to gain water (Klaassen 1996; Bauchinger and Biebach 1998; Jenni and Jenni-Eiermann 1998; Gerson and Guglielmo 2011) and the primary sources of this tissue protein are likely the splanchnic organs and pectoral muscle (see Mizrahy et al. 2011). In our experiment, both treatment groups lost equivalent flight muscle mass overnight, suggesting that water-deprived birds on stopover did not

preferentially catabolize this tissue to produce metabolic water. Given the much higher protein turnover rates of splanchnic organs compared to pectoral muscle in songbirds (Bauchinger and McWilliams 2010), catabolism of the digestive tract during migratory flights may have provided Garden Warblers in our study an important source of metabolic water under dehydrating conditions, as also indicated by recent studies of Blackcaps (*Sylvia atricapilla*; Mizrahy et al. 2011). Our results suggest that the strategy of water-deprived birds may be to conserve pectoral mass as if they had access to drinking water, and increase departure likelihood in search of free water elsewhere as long as they also have sufficient fat stores. Indeed, flight muscle mass at capture—a more integrated and quantitative measure of muscle size than muscle score—apparently did not influence the level of Zugunruhe for either water-deprived or watered birds.

There are many physiological reasons why a migrating bird with adequate energy stores should be eager to leave a stopover site that does not offer free water and food, and stay at one that does. Drinking water on stopover is important to rebuilding digestive capacity, gaining lean and fat mass, and maintaining adequate water intake when choosing high-energy, low-water foods (Sapir et al. 2004; Tsurim et al. 2008; Mizrahy et al. 2011). Essentially, birds require preformed water (e.g., drinking water or water in food) to refuel adequately on stopover, and having access to that water may even contribute to the pace of their migration. Both captive studies and those with free-living birds have shown that migrating passerines with access to water exhibit higher fuel deposition rates than individuals without water through several mechanisms: free water hastens the rebuilding of a digestive tract that was catabolized

during fasting and long-distance flights (Mizrahy et al. 2011), and the birds' dietary choices are in part constrained by the water content of available food items (Sapir et al. 2004; Tsurim et al. 2008). Our experiment uniquely complements these migratory-stopover feeding studies by showing that the availability of drinking water also affects the condition dependency of Zugunruhe behavior. A bird without access to drinking water at a stopover site may be expected to refuel at a slow rate, in the absence of foods with high water content, and, therefore, would benefit from choosing to leave that site, if able. Fuel deposition rates are critical to the pace of migration and distribution of migratory birds at stopover sites, particularly in spring when birds maximize fuel deposition to minimize migration time to breeding grounds (Hedenström and Alerstam 1997; Schaub and Jenni 2000). Therefore, free water's facilitation of fueling should be an important aspect of stopover site selection.

We expect the relevance of water availability to stopover decisions to be greatest in arid conditions and near ecological barriers (Fogden 1972; Carmi et al. 1992; Leberg et al. 1996). Given that migrants crossing ecological barriers may be water-limited rather than fat-limited (e.g., Leberg et al. 1996), recouping or preventing further water losses should be a focal concern at subsequent stopovers. Choosing stopover sites based on water availability likely falls among the many behavioral strategies that birds adopt to avoid dehydration during desert crossings, e.g., landing in oases, seeking microhabitats with shade, avoiding daytime flight, and choosing flight altitudes that minimize evaporative loss (Biebach 1985; Carmi et al. 1992; Klaassen 1996, 2004).

Using deuterium to estimate body composition of warblers: the problem with dehydration

We used the deuterium dilution method to estimate fat mass of Garden Warblers in addition to measuring subcutaneous fat score. We expected the two variables to consistently correlate strongly with each other, but instead found that the correlation between estimated fat mass and fat score was strong only among birds that had access to drinking water. Furthermore, the deuterium injection method resulted in low deuterium concentrations as measured in microdistilled blood water, and consequently biased-high water space estimates and biased-low fat mass estimates in ten of 29 water-deprived birds as opposed to only three of 32 birds that were provided water. We suggest the following potential mechanism as the most likely to explain why measurement of fat mass via the deuterium dilution method was ineffective for dehydrated individuals: the deuterium we injected into the birds may not have distributed evenly between blood and tissue in dehydrated individuals. Songbirds, and birds of other orders, that have lost water mass via flight or heat stress continue to maintain high plasma volume despite dehydration of other tissues (Dawson et al. 1983; Carmi et al. 1993). The D_2O we injected into water-deprived animals may have been preferentially absorbed and retained by dehydrated muscle or other tissues, over the plasma, which we sampled. This unequal distribution among sub-pools of the total body-water pool (Lifson and McClintock 1966; Speakman 1997), and failure to equilibrate regardless of adequate time, could account for the low concentration of

deuterium that we observed in circulation. We, therefore, caution researchers against using the deuterium dilution method in dehydrated individuals.

Conclusions and future directions

Our data suggest that availability of drinking water after sustained flight over an ecological barrier is an important determinant of departure likelihood from a stopover site. Our experiment supports the notion that fat stores affect Zugunruhe levels in migrating songbirds, but adds that access to drinking water changes the condition dependency of migratory restlessness. Water-deprived birds displayed particularly high nocturnal activity levels when they also had high fat scores. These results suggest that access to drinking water, in addition to energy stores, may significantly impact a migrating bird's decision to depart a stopover site if they are dehydrated. Birds crossing the Mediterranean Sea likely arrive at the volcanic island of Ponza in a dehydrated state, and, consequently, individuals with large fat scores may be expected, regardless of muscle size, to depart this relatively dry site more readily than they would leave a site with more freely available water.

We recommend that future studies take advantage of techniques such as quantitative magnetic resonance and radio-telemetry to establish the physiological mechanisms and prevalence of water-based stopover decisions in free-living birds. Magnetic resonance can be used to quantify the total body water as well as lean and fat mass of free-living songbirds with high accuracy (Guglielmo et al. 2011; McWilliams and Whitman 2013). Researchers might adopt a similar approach to that

of Smith and McWilliams (2014) by directly manipulating and quantifying the total body water (as well as lean and fat mass) of birds on stopover and subsequently monitoring their movements and departure dates subsequent to release. Provision of water troughs on the landscape (after Sapir et al. 2004) in concert with this monitoring might further help elucidate the water-based choices of migrants on stopover.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Approval

All applicable international, national, and/or institutional guidelines for the care and use of animals and animal samples were followed.

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Table 3.1. Spearman correlations between two intervals of Zugunruhe and several measures of body condition at capture for Garden Warblers that did not, or did, receive water. Correlations with $p < 0.05$ are in bold.

	No water provided			Water provided		
	n	*r*	*p* value	*n*	*r*	*p* value
Early-night Zugunruhe						
Body mass	**29**	**0.500**	**0.006**	**32**	**0.358**	**0.044**
Fat score	**29**	**0.495**	**0.006**	**32**	**0.604**	**<0.001**
Muscle score	29	0.350	0.063	**32**	**0.362**	**0.042**
Flight muscle mass[a]	29	–0.054	0.781	32	0.170	0.352
All-night Zugunruhe						
Body mass	**29**	**0.388**	**0.037**	32	0.275	0.128
Fat score	29	0.325	0.086	**32**	**0.559**	**0.001**
Muscle score	29	0.284	0.136	**32**	**0.391**	**0.027**
Flight muscle mass[a]	29	0.028	0.883	32	0.161	0.378

[a] Calculated from muscle shape, body mass, and tarsus length at capture, after Bauchinger et al. (2011). Equation used: flight muscle mass = –1.212 + (0.293 x muscle shape) + (0.045 x body mass) + (0.199 x tarsus length).

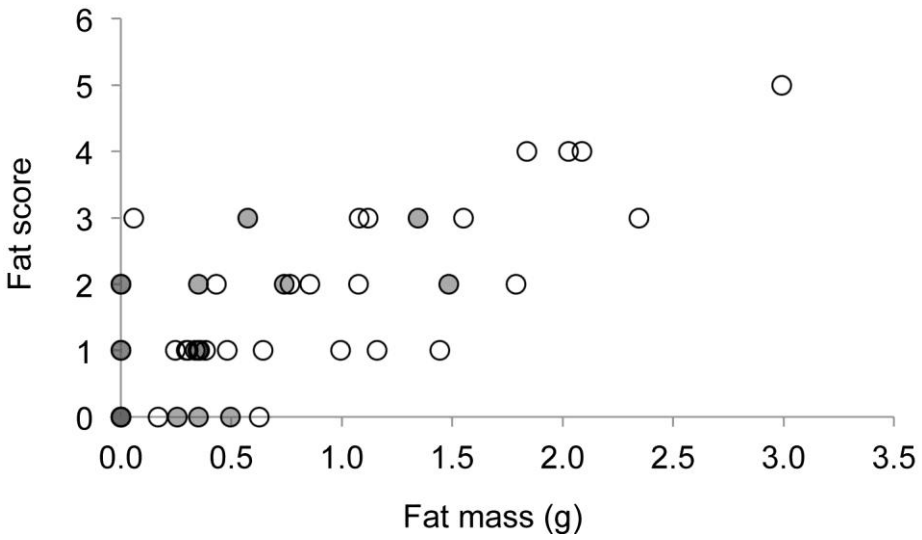

Fig. 3.1 Fat mass estimated by deuterium dilution and fat score estimated visually were strongly correlated among Garden Warblers provided with water (*open circles*) but only weakly correlated among birds that were not provided with water (*filled circles*)

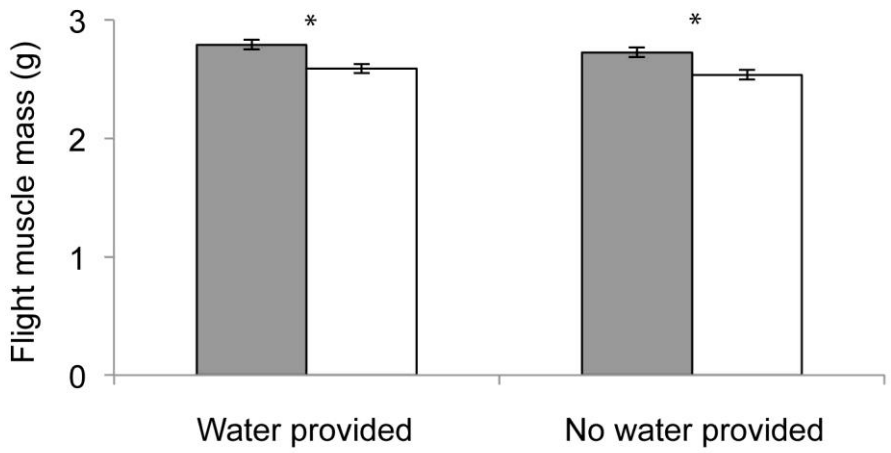

Fig. 3.2 Values of flight muscle mass (g, LS means ± SE) for Garden Warblers decreased from at capture (*filled bars*) to after an overnight fast (*open bars*) for birds provided water as well as birds not provided water

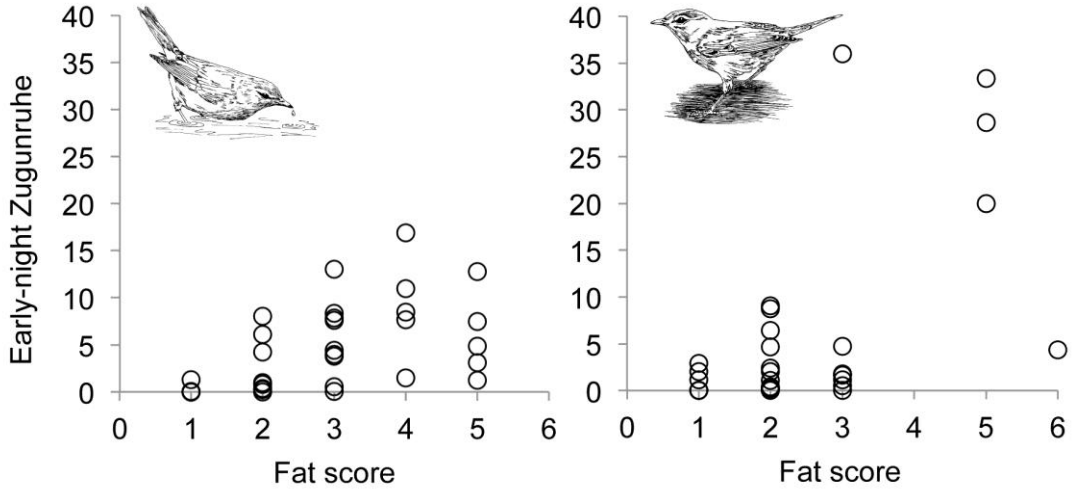

Fig. 3.3 Scatterplots between fat score at capture and early-night Zugunruhe for Garden Warblers that received water (n = 32, *left panel*) versus those that did not receive water (n = 29, *right panel*)

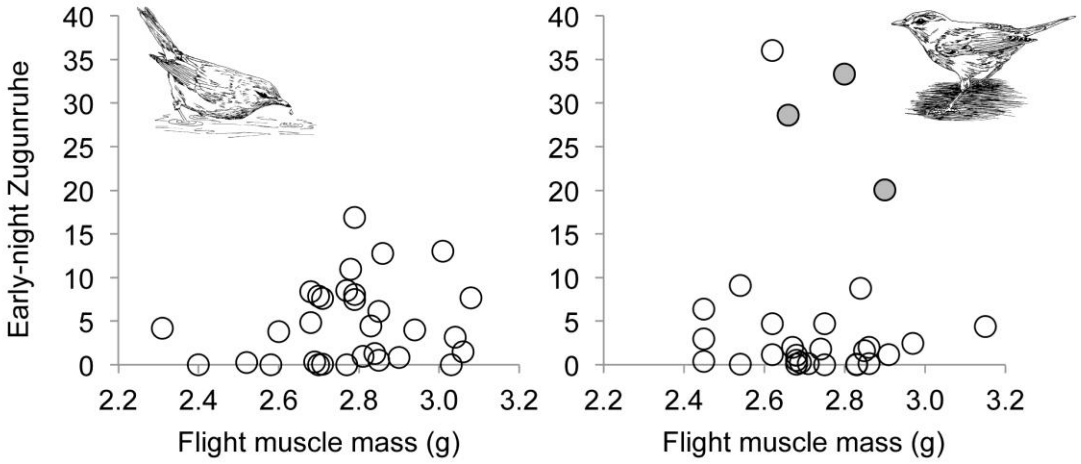

Fig. 3.4 Scatterplots between flight muscle mass at capture and early-night Zugunruhe for Garden Warblers that received water (n = 32, *left panel*) versus those that did not receive water (n = 29, *right panel*, with fat scores of 5 *shaded*)

CHAPTER 4

Dietary antioxidants and flight exercise affect how female birds allocate nutrients to eggs: how carry-over effects work

by

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Key words: antioxidant capacity, lipid oxidation, repeated measures, reproduction, songbirds

Summary statement: Our experiment demonstrates for the first time effects of water-soluble dietary antioxidant supplementation and flight exercise on the composition of songbird eggs, with important implications for wild migratory birds.

Abstract:

Physiological challenges during one part of the annual cycle can carry over and affect performance at a subsequent phase, and antioxidants could be one mediator of trade-offs between phases. We performed a controlled experiment with zebra finches to examine how songbirds use nutrition to manage trade-offs in antioxidant allocation between endurance flight and subsequent reproduction. Our treatment groups included (1) non-supplemented non-exercised group (control group) fed a standard diet with no exercise beyond that experienced during normal activity in an aviary; (2) supplemented non-exercised group fed a water- and lipid-soluble antioxidant-supplemented diet with no exercise; (3) non-supplemented exercised group fed a standard diet and trained to perform daily endurance flight for 6 wk; (4) supplemented exercised group fed an antioxidant-supplemented diet and trained to perform daily flight for 6 wk. After flight training, birds were paired within treatment groups for breeding. We analyzed eggs for lutein and vitamin E concentrations and the plasma of parents throughout the experiment for non-enzymatic antioxidant capacity and oxidative damage. Exercised birds had higher oxidative damage levels than non-

exercised birds after flight training, despite supplementation with dietary antioxidants. Supplementation with water-soluble antioxidants decreased the deposition of lipid-soluble antioxidants to eggs and yolk size. Flight exercise also lowered deposition of lutein, but not vitamin E, to eggs. These findings have important implications for future studies of wild birds during migration and other oxidative challenges.

Introduction

Physiological challenges during one part of the annual cycle (e.g., spring migration) can carry over and affect performance at a subsequent phase (e.g., reproduction; Ebbinge and Spaans, 1995; Bauchinger et al., 2009; Legagneux et al., 2012), although the direct mechanisms that produce these carry-over effects are only rarely understood (Harrison et al., 2011). A particularly interesting but understudied potential mechanism for carry over effects that impact reproductive performance concerns the antioxidant system and its ability to quench pro-oxidants. The general concept is that antioxidants provided to embryos are beneficial (Saino et al., 2003; McGraw et al., 2005; Berthouly et al., 2007, 2008; Marri and Richner, 2014; Jacob et al., 2015); however, if antioxidant supply is limited and requirements prior to reproduction are relatively high, then this may in turn restrict a female's allocation of antioxidants to reproduction and thus produce an important carry-over effect.

Birds rely on suites of antioxidant compounds, both ingested and internally generated, to intercept and neutralize pro-oxidants before they can cause oxidative damage. Evidence suggests that wild birds seek out and acquire dietary antioxidants

while migrating (Alan et al., 2013; Bolser et al., 2013), using available resources on stopover sites to increase their own antioxidant capacity in preparation for their flights (Skrip et al., 2015). Any surplus antioxidants that remain after migration might be deposited to eggs upon arrival to breeding grounds, but no research has investigated this carry-over phenomenon. Migratory birds typically begin breeding shortly after spring migration, and the endurance flights they recently performed might impact their oxidative state (e.g., Skrip et al., 2015) and ability to allocate antioxidants. Although many studies have examined how maternal diet affects deposition of antioxidants to eggs (e.g., Royle et al, 2003; McGraw et al., 2005; Blount et al., 2006), none have considered how using antioxidants during flight could affect subsequent investment of antioxidants during reproduction, or evaluated the impacts of water-soluble dietary antioxidants on songbird egg composition. Previous studies of egg composition and flight performance have focused on lipid-soluble carotenoids and vitamin E. Plasma levels of carotenoids in birds can relate to dietary intake (Koutsos et al., 2003), and vitamin E is considered especially important to avian embryonic development (Surai, 2002). Water-soluble polyphenolic compounds such as anthocyanins, however, are powerful antioxidants with high concentrations in fruits consumed by wild birds (Alan et al., 2013; Bolser et al., 2013), and their contribution to avian antioxidant capacity is emerging as an important area of research (Catoni et al., 2008; Schaefer et al., 2008; Beaulieu and Schaefer, 2013, 2014). Dietary anthocyanins circulate in avian plasma and can help songbirds mount an immune response (Catoni et al., 2008); birds may even select fruits based on their anthocyanin content, using the pigmentation of these

antioxidants as a visual signal of their benefits (Schaefer et al., 2008; Bolser et al., 2013).

We performed a controlled experiment with zebra finches (*Taeniopygia guttata* Vieillot) to determine the combined effects of food chemistry—specifically a blend of lipid- and water-soluble dietary antioxidants—and the demands of flight on allocation of nutrients to eggs in songbirds. This is the first experiment to combine fat- and water-soluble antioxidants in a supplement for songbirds and to test for an effect of dietary antioxidants and long-duration flight exercise on egg composition. Although not migratory, the zebra finch offers many opportunities for mechanistic studies and is used often as a model organism, given that the species breeds readily in the laboratory and lends itself easily to experimental treatments that cannot be accomplished with wild birds (Griffith and Buchanan, 2010). Studies using zebra finches have already shown that diet, body condition, and mate attractiveness affect antioxidant deposition in eggs (Royle et al., 2003; McGraw et al., 2005; Williamson et al., 2006); that egg composition and the diet of young birds affects their antioxidant capacity later in life (Blount et al., 2003; McGraw et al., 2005); and that antioxidant supplementation to the diet improves exercise performance in adult birds (Blount and Matheson, 2006). Zebra finch eggs that contain high antioxidant levels also have low concentrations of pro-oxidants, are more likely to hatch, and produce young more likely to survive the nestling stage (McGraw et al., 2005).

In this experiment we tested the following hypotheses related to the impacts of dietary antioxidants and exercise on the circulating oxidative status of adult songbirds and allocation of antioxidants to eggs: (Hypothesis 1) Endurance flight poses an

oxidative challenge. We expected exercised birds to have higher circulating oxidative damage levels after flight training than non-exercised birds. Several studies have associated long-distance flight with oxidative damage in free-flying birds (trained pigeons [*Columba livia* Gmelin], Costantini et al., 2008; migrating European robins [*Erithacus rubecula* Linnaeus], Jenni-Eiermann et al., 2014) and acute flight bouts with oxidative damage in captive birds (zebra finches, Costantini et al., 2013); our results would support in a controlled setting that endurance flight poses an oxidative challenge to songbirds. (Hypothesis 2) Dietary antioxidants contribute to antioxidant protection in birds. We expected supplemented birds to display higher antioxidant capacity and lower oxidative damage than non-supplemented birds. If birds build antioxidant capacity by acquiring dietary antioxidants (as suggested by Beaulieu and Schaefer, 2014 and Skrip et al., 2015), our results would support a mechanism for antioxidant protection. (Hypothesis 3) Flight and diet exert effects on subsequent breeding. Based on feeding studies by other researchers who used solely lipid-soluble dietary antioxidants (e.g., McGraw et al., 2005), we expected that supplemented birds would lay eggs with higher lipid-soluble antioxidant concentrations than non-supplemented birds. We also expected that exercised birds would lay eggs with lower antioxidant concentrations than non-exercised birds, given that available antioxidants should be used during flight. We expected an interaction, however, between diet and exercise treatments, predicting that supplementation would prevent lower allocation of antioxidants among exercised birds.

Materials and methods

All captive work was approved by the University of Rhode Island Institutional Animal Care and Use Committee (IACUC # AN11-12-009). Our experimental design was 2 × 2 factorial, with two diet treatments and two exercise treatments among four groups of adult zebra finches: (1) non-supplemented non-exercised group (control group) fed a standard diet with no exercise beyond that experienced during normal activity in an aviary; (2) supplemented non-exercised group fed an antioxidant-supplemented diet with no exercise; (3) non-supplemented exercised group fed a standard diet and trained to perform daily endurance flight; (4) supplemented exercised group fed an antioxidant-supplemented diet and trained to perform daily flight. The timeline of the experiment consisted of a 4-mo diet acclimation period followed by three sequential phases: a 4-wk diet supplementation phase, 6-wk exercise phase, and a breeding phase (Fig. 1). During the diet supplementation phase and exercise phase, we housed zebra finches in four same-sex indoor aviaries (2.1 × 0.9 × 1.8 m, L × W × H, initial stocking = 19 – 21 birds per aviary) on a 14:10 h light:dark cycle under full spectrum light, with lights on at 0600. During the breeding phase, we housed mating pairs in individual cages (0.6 × 0.4 × 0.35 m, L × W × H) with nesting material.

Phase I — Diet treatment

For 4 mo before the diet phase began, all birds were provided daily *ad libitum* water, cuttlebone, grit, and standard seed mixture (Kaytee® Supreme™ Finch mix), plus kale

twice weekly, to acclimate them to the lab environment. Half of the birds (two of the same-sex aviaries; n = 17 females and 19 males) remained on this non-supplemented diet while the other birds were assigned to a supplemented (high-antioxidant) diet (n = 15 females and 17 males) on which they were maintained for the subsequent 4-wk diet supplementation phase of the experiment. All birds remained on their respective diets for the remainder of the experiment (through the exercise and breeding phases).

The supplemented group received cuttlebone, grit, the standard seed mixture, and kale twice weekly, plus *ad libitum* water supplemented with both lipid-soluble antioxidants (4.0 µg FloraGLO 10% lutein beadlets per mL; DSM Nutritional Products Ltd., Parsippany, NJ) and water-soluble antioxidants (3.2 mg Standard Elderberry Powder 25% per mL; Artemis International, Inc., Fort Wayne, IN). The elderberry powder was produced by spray-drying elderberry extract and standardized to a minimum of 25 g/100 g anthocyanin, expressed as cyanidin-3-glucoside on a maltodextrin excipient; the powder also contained > 40 g/ 100 g polyphenols, expressed as catechin (Artemis International, Inc., Fort Wayne, IN). Thus, our water-soluble supplementation consisted of two types of flavonoids—catechins and anthocyanins.

The concentration of lutein was chosen based on the average seed and water consumption of the study animals, to attain dosages used by other researchers using zebra finches (McGraw et al., 2004, 2005). These authors evaluated the lutein content of seed and raised the dosage to presumably the upper limit of what birds might naturally encounter (McGraw et al., 2001, 2004). Lutein is a lipid-soluble antioxidant, found in seed and a pigment in both beaks and feathers (McGraw et al., 2004). It is

one of the main carotenoids in the finches' seed diet (McGraw et al., 2001). McGraw et al. (2005) used daily consumption rates of seed and water to estimate that birds derived ca. 15 µg lutein/d from seed (Kaytee® Forti-Finch™ mix) and ca. 18 µg lutein/d from supplementation to the drinking water, for a total of ca. 33 µg lutein/d/bird. Based on measurement of seed (3.5 g/d) and water (3.5 mL/d) consumption of finches in our study, we estimated that the birds derived ca. 18 µg lutein/d from seed (Kaytee® Supreme™ Finch mix) and ca. 14 µg lutein/d from supplementation to the drinking water for a total of ca. 32 µg lutein/d/bird.

We chose the anthocyanin concentration to approximate that used by researchers studying the effect of anthocyanin supplementation on food choice and immunocompetence in European blackcaps (*Sylvia atricapilla* Linnaeus; Catoni et al., 2008; Schaefer et al., 2008). These blackcaps consumed 2.8 mg of anthocyanins/d, in the form of a standardized 50% anthocyanin extract; the researchers based their dose on "estimates of flavonoid ingestion by Blackcaps during periods of relatively low frugivory (<10 berries/d)" (Catoni et al., 2008:651). We calculated a similar dosage with a standardized 25% extract and based the delivered concentration on an average daily water consumption of 3.5 mL/d.

We chose to use a 4-wk diet supplementation period before beginning flight training, assuming that this interval was long enough to affect the antioxidant capacity of zebra finches. McGraw et al. (2004) demonstrated changes in zebra finch beak color (indicating that carotenoids were assimilated in tissue) using 4 wk of carotenoid supplementation. McGraw et al. (2005) used a 3-wk diet treatment to find that zebra finches fed a carotenoid-supplemented diet produced eggs with higher carotenoid

content and with better resistance to pro-oxidants; supplemented females also produced more sons with deeply pigmented beaks (indicative of high carotenoid content) than those on a non-supplemented diet.

Blount and Matheson (2006) kept zebra finches on a carotenoid-supplemented diet for 8 wk before measuring flight performance and found that the supplementation improved flight times from an escape chamber; the duration of the diet treatment was not justified in their paper, and no studies have quantified the length of time necessary for an antioxidant-rich diet to have an impact on bird flight performance. Larcombe et al. (2010) kept budgerigar parrots (*Melopsittacus undulates* Shaw) on a high- or low-antioxidant diet for 12 mo before their flight training experiments. Given that migratory birds consume dietary antioxidants at stopover sites (Bolser et al., 2013) where they usually reside for days to at most weeks, the benefits of antioxidant consumption would have to be relatively immediate to be useful during migration.

Phase II — Exercise treatment

The exercise phase consisted of daily flight in an arena modeled on Bauchinger et al. (2010), with horizontal perch-to-perch flight totaling 6.4 km/d. Briefly, we constructed a soft-sided enclosure 5.48 × 1.83 × 2.13 m (L × W × H), with a soft partition lengthwise down the center. A human observer continuously walked around the partition for 1 h, herding the flock of 32 birds from one perch to the other and keeping track of the number of circuits (n = 300 circuits/h) with a hand counter. Perch-to-perch flight is an accepted method of exercise for captive zebra finches (e.g., Bauchinger et

al., 2010; Costantini et al., 2013), with repeated takeoffs and short flights requiring three times the energy of sustained flapping flight (Nudds and Bryant, 2000).

Half of the birds in each diet group (half of the birds in each same-sex aviary) were randomly assigned to exercise training. Each morning, we used custom-designed capture boxes in the aviaries to retrieve the birds in the exercised group for training. Birds voluntarily flew into the boxes and were captured or released through a small door; therefore, both exercised and non-exercised birds experienced similar handling. Exercised birds were flown every day in two 1-h sessions (starting at 1100 and 1330), 7 d/wk for 6 wk, totaling 270 km of flight for each exercised bird. Non-exercised birds remained in the aviaries while conspecifics were exercised, although food and water were removed from the aviaries so that temporal access to food, water, and dietary antioxidants was the same for exercised and non-exercised birds. After the last circuit of a flight session, a blind was lowered to block off one side of the arena, a small trap door was opened at one end, and the birds flew into a small cage on the other side of the trap door; they were then sorted back into their same-sex diet groups. During the 1-h break between the two 1-h-long flight sessions each day, all birds were provided seed and water. Exercised birds were returned to the aviaries after their second flight each day.

Phase III — Breeding

After the flight phase ended on 24 August 2012, we randomly assigned birds within the four treatment groups into mating pairs and housed them individually for breeding.

We checked the nests of breeding birds once daily, and eggs were collected as laid and replaced with plastic dummy eggs. Each egg was weighed, then stored at –20ºC until analyzed for antioxidant content (Royle et al., 2003; Gil et al., 2004; Rutstein et al., 2004; Blount et al., 2006; Sandell et al., 2007). Most clutches were laid between 31 August and 11 October 2012, with one clutch completed on 3 November; there was no pattern in the timing of clutch completion by treatment.

Blood sampling and analysis

We drew blood samples three times from the same individuals during the experiment to track plasma oxidative measures in response to treatments: once after the 4-wk diet supplementation phase and before the exercise phase, once after the 6-wk exercise phase and before breeding, and once after completion of a clutch (Fig. 1); we considered a clutch complete if the female produced no more eggs for five days after collection of the final egg (Sandell et al., 2007). All blood samples (150 µl) were collected within 12 minutes after entering the room at 0800 (or between chiefly 0800 and 1000 during the breeding phase) to control for any acute changes in plasma oxidative measures associated with disturbance and handling. Blood from the brachial vein was collected into heparinized capillary tubes, centrifuged for 6 min at 10,000 rpm, and plasma was stored at –80ºC until biochemical analysis.

We determined circulating antioxidant capacity as the ability of a plasma sample to neutralize an oxidizing assault of hypochlorous acid, using the OXY-Adsorbent Test (concentration unit = mmol/L of HClO neutralized; Diacron

International, Grosseto, Italy); and measured oxidative damage as the presence of circulating hydroperoxides, which include products of lipid oxidation, using the d-ROMs test (concentration unit = mmol/L H_2O_2 equivalents; Diacron International, Grosseto, Italy; see Costantini et al., 2007 for further details). We chose the OXY and d-ROMs tests as general whole-animal markers of circulating oxidative status, and to facilitate comparison of results with previous studies. We anticipated that OXY, as a measure of plasma non-enzymatic antioxidant capacity, would be particularly relevant in birds acquiring dietary antioxidants (Beaulieu and Schaefer, 2014); the d-ROMs test provides an index of damage to fats and has been shown to increase in exercised pigeons after flights (Costantini et al., 2008) and to decrease with time on stopover after flights (Skrip et al., 2015). We performed all metabolite assays using a microplate spectrophotometer (Biotek Powerwave 340, Winooski, VT). We ran samples in duplicate unless a coefficient of variation > 15% was observed, in which case we ran a third replicate if sufficient plasma was available.

We present results from parents that produced complete clutches and for which plasma was available for all three sampling periods. One male and two females were not sampled in the before training phase and were therefore excluded from blood analysis; plasma volumes for some individuals were inadequate for performing both assays for one time period, in which cases the OXY test was prioritized. Data from 37 individuals were therefore available for analysis of the OXY-Adsorbent Test results (n = 10 [5 male, 5 female] for non-supplemented non-exercised, n = 9 [5 male, 4 female] for supplemented non-exercised, n = 10 [5 male, 5 female] for non-supplemented exercised, n = 8 [4 male, 4 female] for supplemented exercised). Data from 30

individuals were available for analysis of d-ROMs results (n = 7 [5 male, 2 female] for non-supplemented non-exercised, n = 7 [4 male, 3 female] for supplemented non-exercised, n = 9 [5 male, 4 female] for non-supplemented exercised, n = 7 [2 male, 5 female] for supplemented exercised).

Liver sampling and analysis

After the exercise phase, we euthanized by cervical dislocation 28 birds that were randomly assigned to not be part of the breeding phase, to determine the activities of three antioxidant enzymes in liver tissue (nmol enzyme/min/mg of protein). We determined total protein in liver samples using the Bio-Rad Bradford Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA) and used commercial kits (Cayman Chemical Company, Ann Arbor, MI) to measure the activity of glutathione peroxidase, catalase, and superoxide dismutase (n = 6 [3 male, 3 female] for non-supplemented non-exercised, n = 8 [5 male, 3 female] for supplemented non-exercised, n = 8 [5 male, 3 female] for non-supplemented exercised, n = 6 [3 male, 3 female] for supplemented exercised).

Egg sampling and analysis

We determined concentrations of lutein and vitamin E (α-tocopherol) in yolk using reversed phase high performance liquid chromatography (RP-HPLC), generally following the methods of Williamson et al. (2006). We dissected eggs from frozen and

weighed yolks and albumen on a digital balance to the nearest 0.0001 g. For each egg, we combined 100 mg of yolk with 0.7 mL 5% NaCl solution, 1 mL ethanol, and 2 mL hexane, homogenized the mixture for 30 s, and centrifuged for 5 min at 2,000 rpm. We then collected the hexane phase containing vitamin E and carotenoids, and extracted with hexane again before drying the combined collection under vacuum in a SPD1010 SpeedVac© System. We dissolved the remaining residue in 500 µL of methanol/dichloromethane (1:1 v/v), ready for RP-HPLC analyses, which were performed on a Hitachi Elite LaChrom system consisting of a L2130 pump, L-2200 autosampler, and a L-2455 Diode Array Detector all operated by EZChrom Elite software (Agilent Technologies, Inc., Pleasanton, CA). We determined lutein and vitamin E concentrations using a Waters Spherisorb S5ODS2, 5-µm reverse-phase column, 25 cm x 4.6 mm (Sigma Aldrich, St. Louis, MO) with a mobile phase of methanol/water (9:1 v/v) at a flow rate of 1 mL min^{-1}. Lutein was detected as single peak at 445 nm and vitamin E at 290 nm; peaks were identified by comparison with standards.

We collected complete clutches from 20 pairs of birds, although three of those birds (see above) were not blood sampled in the before training period (n = 6 clutches for non-supplemented non-exercised, n = 5 for supplemented non-exercised, n = 5 for non-supplemented exercised, n = 4 for supplemented exercised).

Statistical analysis

All analyses were performed using SAS 9.4 software (SAS Institute, 2014).

Blood oxidative status

We performed repeated measures analysis of variance (RMANOVA) to determine the effects of diet and exercise treatments, and their interaction, on plasma measures of oxidative status over the course of the experiment. We also performed tests of effect slices to detect treatment group differences within or between time periods. Antioxidant capacity and oxidative damage data from three time periods were used: before flight training, after flight training (and before breeding), and after breeding. Data from 37 individuals were available for antioxidant capacity analyses, and data from 30 individuals were available for oxidative damage. The compound symmetry (CS) covariance structure produced the best model fit for antioxidant capacity, as determined by AIC, and the heterogeneous autoregressive [arh(1)] covariance structure provided the best model fit for oxidative damage. We checked model residuals for homogeneity of variance and normality to verify satisfaction of model assumptions.

Liver antioxidant enzymes

We performed two-way ANOVAs with an interaction term to determine the effects of diet and exercise treatments on liver enzyme activities. We checked model residuals for homogeneity of variance and normality to verify satisfaction of model assumptions.

Egg composition

In the wild, a typical clutch size for the zebra finch is 5 eggs (Zann, 1996); most pairs in our study (n = 14) laid 5 or fewer eggs, but 2 pairs laid 8 eggs, 1 pair laid 9, and 3 pairs laid 6; given that there were too few pairs that laid more than 5 eggs, we truncated our analysis dataset to exclude these later-laid eggs (13 eggs from 6 pairs).

We used mixed general linear models to assess differences between treatment groups and across laying order for the following response variables: egg mass, proportion of yolk, and concentration of yolk antioxidants. Mixed linear models allow the inclusion of random factors; here, we modeled random slopes and intercepts for each pair of birds, and nested eggs within pairs, thereby accounting for the uniqueness of pairs and non-independence of eggs within clutches. We further used the Kenward-Roger method to adjust denominator degrees of freedom (SAS Institute, 2014) to avoid pseudoreplication of eggs within clutches. Laying order, diet treatment, and exercise treatment were included as main effects, as well as in two-way interactions and a three-way interaction. We checked model residuals for homogeneity of variance and normality to verify satisfaction of model assumptions.

Results

Effect of diet and exercise on circulating oxidative status

Oxidative damage

Oxidative damage did not vary with phase in the experiment (Time, $F_{2,50} = 0.06$, $p = 0.945$, Table 1), but was higher in males (LS mean ± s.e.m., 40.53 ± 1.47) than females (35.95 ± 1.69; Sex, $F_{1,25} = 4.14$, $P = 0.0525$). Birds in the exercised group (40.25 ± 1.51) had on average slightly higher d-ROMs values than unexercised birds (36.23 ± 1.63; Exercise, $F_{1,25} = 3.27$, $P = 0.0826$), although the only time period for which the difference between groups was statistically significant was after the flight phase (test of effect slices for Exercise × Time; $F_{1,50} = 10.16$, $P = 0.0025$, Fig. 2A). Exercised and non-exercised groups did not differ before training (test of effect slices for Exercise × Time; $F_{1,50} = 0.83$, $P = 0.3660$, Fig. 2A) or after breeding (test of effect slices for Exercise × Time; $F_{1,50} = 0.30$, $P = 0.5850$, Fig. 2A).

Antioxidant capacity

In contrast to oxidative damage, antioxidant capacity varied with phase in the experiment (Time, $F_{2,64} = 3.87$, $P = 0.026$, Table 2), with OXY-Adsorbent test values dropping after the flight phase and returning to pre-flight levels after breeding. A suggestive Exercise × Time interaction (Exercise × Time, $F_{2,64} = 2.84$, $P = 0.066$) indicates that this trend was more evident in birds that did not exercise (Fig. 2B). Furthermore, tests of effect slices show that change over time occurred only in non-exercised birds (test of effect slices for Exercise × Time; $F_{2,64} = 6.82$, $P = 0.0021$, Fig. 2B) and not in exercised birds (test of effect slices for Exercise × Time; $F_{2,64} = 0.09$, $P = 0.9125$, Fig. 2B).

Effect of diet and exercise on liver enzyme activities

Enzyme activities varied widely within treatment groups (coefficient of variation = 15–52%) and were not statistically different between treatments. Diet and exercise had no impact on the activity of liver glutathione peroxidase (global $F_{3,24} = 1.63$, $P = 0.2093$), catalase (global $F_{3,24} = 0.64$, $P = 0.5953$), or superoxide dismutase (global $F_{3,24} = 0.29$, $P = 0.8313$).

Effect of diet and exercise on egg composition

Lutein concentration

Lutein concentration in egg yolk (μg g^{-1}) predictably decreased with laying order, but the rate of change varied by treatment group (Order × Diet × Exercise interaction, $F_{1, 61.7} = 7.82$, $P = 0.0069$, Table 3). Specifically, the non-supplemented, non-exercised group displayed a more negative slope than the other groups, which were similar to each other (post-hoc contrasts, $t > |2|$, $P < 0.02$), and had a higher y-intercept than the other groups, which were similar to each other (post-hoc contrasts, $t > |2|$, $P < 0.02$). That is, pairs in the non-supplemented, non-exercised group had initially higher concentrations of lutein in their eggs and that concentration decreased more sharply over the laying order (Fig. 3A).

Vitamin E concentration

Vitamin E concentration in egg yolk (μg g^{-1}) also decreased with laying order, but the rate of change varied by diet group only (Order × Diet, $F_{1, 61.9} = 10.79$, $P = 0.0017$; Table 4, Fig. 3B). Specifically, the non-supplemented group displayed a more negative slope, and higher y-intercept, than the supplemented group (post-hoc contrasts, $t > |2|$, $P < 0.02$; contrasts between supplemented exercised and supplemented non-exercised, $t = |1.9|$, $P < 0.07$). That is, pairs in the non-supplemented group had initially higher concentrations of vitamin E in their eggs and that concentration decreased more sharply over the laying order (Fig. 3B). Exercise treatment did not affect vitamin E concentration.

Egg mass

Egg mass did not change with laying order for non-supplemented pairs, but increased with laying order for supplemented pairs (Order × Diet, $F_{1, 18.7} = 12.77$, $P = 0.0021$; Table 5; Fig. 3C). Among supplemented birds, egg mass increased at a greater rate in the exercised group than in the non-exercised group (post-hoc specific contrast between slopes, $t_{16.8} = 2.54$, $P = 0.0212$), and eggs laid by exercised, supplemented birds were on average ca. 154 mg (± 68 mg s.e.m.) heavier than those of non-exercised, supplemented birds (post-hoc contrast of LS means, $t_{17} = 2.28$, $P = 0.0356$).

Proportion of yolk in egg

Proportion of yolk did not change with laying order (Order, $F_{1, 25.1} = 1.39$, $P = 0.2491$) or with exercise treatment (Exercise, $F_{1, 38} = 0.31$, $P = 0.5836$), but non-supplemented birds had a greater proportion of yolk in their eggs than supplemented birds (Diet, $F_{1, 38} = 5.27$, $P = 0.0273$). There was also suggestive evidence of a diet × exercise interaction, whereby non-supplemented, non-exercised birds had the greatest proportion of yolk (30%, Table 6) in their eggs (Diet × Exercise, $F_{1, 38} = 3.74$, $P = 0.0606$). No other interactions were significant (Order × Diet, $F_{1,25.1} = 0.00$, $P = 0.9931$; Order × Exercise, $F_{1,25.1} = 0.24$, $P = 0.6277$; Order × Diet × Exercise, $F_{1,25.1} = 3.25$, $P = 0.0835$).

Discussion

For the first time, our experiment demonstrated that antioxidant supplementation and endurance flight together produce effects on songbird reproduction. Our results were consistent with Hypothesis 1 in that oxidative damage was greater in exercised birds compared to non-exercised birds after a 6-wk training period. Our results were not consistent with Hypothesis 2 in that birds fed the dietary antioxidant blend we provided had similar antioxidant capacity compared to birds that were not supplemented. Regarding Hypothesis 3, we found that exercise and diet supplementation both directly affected female allocation of resources to eggs within a clutch, and we provide evidence that simultaneous consumption of water- and lipid-

soluble antioxidants inhibits absorption and thus allocation of lipid-soluble antioxidants to eggs. We discuss the ecological implications of these results, and how carry-over effects have important implications for migratory birds.

Hypothesis 1: Does endurance flight pose an oxidative challenge?

We predicted that exercised birds would have higher circulating oxidative damage levels than non-exercised birds after flight training, based on the hypothesis that flight poses an oxidative challenge. Consistent with this prediction, we found that exercised and non-exercised groups diverged in their damage levels after the 6-wk flight training period, with exercised birds showing greater damage than non-exercised birds. The difference between the two groups is evident only during this time period, due to a sizable decrease in variation within groups. Our data also suggest that male birds endure higher oxidative damage than female birds, which is not surprising, given that estrogen has been shown to have antioxidant effects by increasing levels of other antioxidants (particularly vitamin E) and directly scavenging pro-oxidants (Feingold et al., 1993; Tiidus, 1995; Behl et al., 1997; Halifeoglu et al., 2003).

Oxidative damage has been widely assumed to occur in volant birds after extended periods of exercise, but rarely has it been explicitly demonstrated (Costantini et al., 2008, 2013; Jenni-Eiermann et al., 2014). Even with dietary antioxidant supplementation, the increased metabolic demand of flight appears to have damaged lipids in our study; repairing damage is probably then an unavoidable cost of long flights, with which migratory birds simply must cope. Skrip et al. (2015) showed that

wild garden warblers (*Sylvia borin* Boddaert) on stopover were able to decrease the same oxidative damage marker in a matter of days after crossing the Mediterranean Sea, indicating some ability to recover from oxidative damage associated with long flights.

Hypothesis 2: Do dietary antioxidants increase overall antioxidant protection in birds?

We expected that supplemented birds would have higher circulating non-enzymatic antioxidant capacity and lower oxidative damage than non-supplemented birds, based on the hypothesis that the polyphenols we supplied would increase antioxidant protection, as seen by Beaulieu and Schaefer (2014) for Gouldian finches (*Erythrura gouldiae* Gould). However, our results were not consistent with theirs, perhaps because of the mode of antioxidant delivery. We provided a polyphenol extract dissolved in drinking water rather than polyphenols mixed in food as in previous studies (Catoni et al., 2008; Beaulieu and Schaefer, 2014). Given that the absorption of phenolic compounds can depend on associated sugars (He et al., 2006), it is possible that the absence of a natural food matrix (and associated micro- and macronutient content) impaired absorption of dietary antioxidants in our study.

It is also possible that the antioxidant capacity of our experimental birds was already at an upper limit and therefore could not be increased by supplementation or that antioxidant capacity was maintained by compensatory changes in the contribution of endogenous and dietary antioxidants. The OXY values we observed were greater

than those reported by Beaulieu and Schaefer (2014) and generally higher and less variable than those observed in wild red-eyed vireos (*Vireo olivaceus* Linnaeus) and blackpoll warblers (*Setophaga striata* Forster) preparing for migration at an autumn stopover site (Skrip et al., 2015). The OXY-Adsorbent test accounts for both dietary and endogenous contributions to antioxidant capacity (excluding uric acid), and therefore it is possible that supplemented birds relied less on endogenous micromolecular antioxidants for their antioxidant capacity given the dietary antioxidants we supplied. Determining an animal's reliance on endogenous versus dietary antioxidants would require measuring specific compounds (e.g., glutathione, phenolics, vitamin E) circulating in the plasma (Skrip and McWilliams, 2016).

Both exercised and non-exercised birds had antioxidant capacity and antioxidant enzyme activities similar to each other during all three phases of the experiment, although we did observe that circulating antioxidant capacity dropped in non-exercised birds but not exercised birds during exercise training, based on the tests of effect slices (Fig. 2B). During this time, birds were deprived of food and water for approximately two non-consecutive hours mid-day while training took place. Non-exercised birds maintained their body mass during this time period, while exercised birds lost mass during training and then returned to their pre-training mass after breeding (RMANOVA, Time × Exercise, $F_{2,64} = 3.61$, $P = 0.0327$; data not shown). Lower antioxidant capacity among non-exercised birds, therefore, was not associated with body mass but with some other, unknown, endogenous change.

Hypothesis 3: Do flight and diet affect subsequent egg composition?

Our experiment revealed for the first time that flight exercise and supplementation with a combination of water- and lipid-soluble dietary antioxidants both affect the allocation of lipid-soluble antioxidants in eggs by female songbirds. We found several important effects, but not the ones that we anticipated. Our expectation that birds supplemented with water- and lipid-soluble antioxidants would lay eggs with higher lipid-soluble antioxidant concentrations than non-supplemented birds was not met; indeed, we found the opposite. The notion that exercised birds must expend some of their antioxidant capacity and thus subsequently would lay eggs with lower antioxidant concentrations than non-exercised birds was only partially supported; exercise affected deposition of lutein but not vitamin E.

Determining the effects of exercise and diet supplementation on how females allocate resources to eggs requires careful consideration of how egg composition changes with laying order within a given clutch. Lutein and vitamin E concentrations decreased with laying order, as has been found in many other studies including in captive zebra finches (Royle et al., 2003; Blount et al., 2006; Williamson et al., 2006), wild passerines (barn swallow, *Hirundo rustica* Linnaeus, Saino et al., 2002; red-winged blackbird *Agelaius phoeniceus* Linnaeus, Newbrey et al., 2015) and non-passerines (gulls, Royle et al., 2001). We found, however, that exercise and diet supplementation affected the rate of decrease in egg antioxidants with laying order; that is, the treatments affected the relative amount antioxidants females deposited to each consecutive egg.

Diet effects

Contrary to expectations, we found that dietary supplementation lowered lutein and vitamin E concentrations in eggs, as compared to non-supplemented controls (Fig. 3A,B). Given that we provided lutein in the supplement, we predicted that supplemented birds would deposit greater lutein concentrations in eggs (e.g., McGraw et al., 2005; Royle et al., 2006). We suggest that the reduced lipid-soluble antioxidants deposited in eggs occurred because of a negative effect of water-soluble antioxidants on absorption of lipid-soluble antioxidants. Ours is the first experiment to combine water-soluble antioxidants (i.e., flavonoids, including anthocyanins and catechins) and lipid-soluble antioxidants (i.e., carotenoids including lutein) into one supplement given to songbirds. We did so because free-living birds regularly encounter foods that contain both types of antioxidants (e.g., fruits; Alan et al., 2013). The results of our study are consistent with the hypothesis that the water-soluble flavonoids in the supplement interfered with the absorption of lipids and lipid-soluble antioxidants, and so supplemented birds actually assimilated less of the lipid-soluble antioxidants available in their diet (i.e., not only in the supplement, but also from the standard seed mixture and the kale) than birds that did not receive flavonoids.

Fat-soluble antioxidants like vitamin E and carotenoids are assimilated via micelles formed in the intestine by dietary lipids (Surai, 2002). Past studies in poultry and rats have found that catechins (one sub-class of polyphenolic antioxidants in the extract we provided) interfere with the assimilation of lipids from micelles, thereby lowering cholesterol absorption and causing chickens (*Gallus gallus domesticus*

Linnaeus) to lay eggs with lower yolk cholesterol and yolk lipids (Bravo, 1998; Biswas et al., 2000; Koo and Noh, 2007). This effect may not be restricted to catechins, given that a study with non-flavonoids found similar evidence: When fed resveratrol (a stilbene-type polyphenol), quail (*Coturnix coturnix japonica* Linnaeus) laid eggs with narrower yolks than non-resveratrol-fed birds; yolks were narrower as supplementation increased from 200 to 400 mg/kg (Sahin et al., 2010). We found that water-soluble antioxidant (i.e., flavonoid) supplementation decreased deposition of both vitamin E and lutein, and furthermore that such supplementation decreased the proportion of yolk in eggs; this evidence supports the idea that supplementation with water-soluble antioxidants lowered the amount of fat-soluble compounds that could be deposited to eggs.

Although wild birds encounter both water- and lipid-soluble antioxidants in their diet, and often in the same food items (e.g., fruits; Alan et al., 2013), it may behoove them to be choosy about the timing of polyphenol consumption, or to make sure that they consume enough fat to compensate for any inhibitory effects on absorption. Beaulieu and Schaefer (2014) found that captive Gouldian finches chose to consume calorie-poor seeds rich in polyphenolic antioxidants during the middle of the day, and calorie-rich, polyphenolic-poor seeds during the morning and evening; the researchers concluded that the birds were free to increase their antioxidant capacity only when their caloric needs were satisfied. Our study may provide further insight into their results, by suggesting that songbirds may better assimilate calories when not simultaneously consuming polyphenols and that these birds may be capable of making the best temporal dietary decisions when presented a choice. Further experiments

would be necessary to tease out by what percentage polyphenols lower assimilation efficiency of fat-soluble compounds and how consumption of different types of antioxidants affect birds' dietary decisions, but it's apparent that the polyphenols we supplied in the drinking water affected how zebra finches were able to allocate lutein and vitamin E to tissues.

For the first time in songbirds, we have shown that consumption of water-soluble polyphenols decreased allocation of lipid-soluble antioxidants (i.e., lutein and vitamin E) in eggs, presumably because those polyphenols interfere with the absorption of lipid-soluble antioxidants in the intestine. This finding has important implications for migratory birds consuming polyphenols along with fat-soluble antioxidants on stopover sites or wintering grounds; our results suggest that at least some sub-classes of polyphenols do not provide an additive antioxidant benefit when consumed—because they hinder absorption of fat-soluble antioxidants and fats themselves. Clearly, complicated interactions occur among molecules during digestion, and our findings should help researchers think more deeply about what actual nutrition birds receive from the fruits and other foods they eat, depending on their composition.

Exercise effects

Our experiment also demonstrated that flight exercise lowered lutein, but not vitamin E, deposition in eggs. Among non-supplemented birds, lutein concentration in eggs was clearly lower for exercised birds than non-exercised controls (Fig. 3A). We

predicted that exercised birds would deposit fewer antioxidants to eggs because fewer should be available after the oxidative challenge of flight, but we did not anticipate a difference in deposition depending on the compound.

There are two possible, non-mutually exclusive, explanations for the disparity between exercise effects on lutein and vitamin E: (1) It is very likely that by burning fat to fly, the exercised birds in our experiment reduced lutein stores available to then deposit into their eggs. In birds, vitamin E is largely stored in the liver, whereas lutein is stored in subcutaneous body fat, and can be mobilized from that fat in times of need (Metzger and Bairlein, 2011). A female zebra finch's fat score is predictive of the lutein concentration in her eggs but not the vitamin E concentration (Williamson et al., 2006), demonstrating the importance of body fat stores to lutein deposition in eggs. We did not measure changes in fat stores in this study, but it is well accepted that birds, unlike other exercising vertebrates, rely primarily on fats for fuel. (2) Although carotenoids and vitamin E work together to protect lipids during an oxidative challenge, oxidized vitamin E can be recycled to its active form by carotenoids and other compounds, whereas carotenoids may take on a pro-oxidant nature (Surai, 2002). It is possible that the oxidative challenge of flight eliminated carotenoids such as lutein, but not vitamin E, from exercised birds' antioxidant reservoirs, reducing their availability for deposition. Carotenoids are important contributors to sexual signaling in zebra finches (McGraw et al., 2004), and mate attractiveness can influence maternal investment in eggs (e.g., Williamson et al., 2006). At the time of breeding in our experiment, however, we found no statistical difference in beak

redness between treatment groups (as measured by portable reflectance spectrophotometer; data not shown).

Although zebra finches are not migratory, our exercise findings have important implications for studies of migratory birds, which have similar mechanisms of antioxidant storage and lipid metabolism. Researchers commonly consider leftover fat stores advantageous to female songbirds after spring migration, given that greater energy stores enable them to overcome food shortages or potentially produce higher-quality eggs. Our data suggest that female birds with high residual fat stores may also be better able to provision their eggs with lutein, an important antioxidant, given that lutein is stored in body fat (Metzger and Bairlein, 2011). We therefore hypothesize that wild female birds might accumulate both fat-soluble antioxidants and lipid stores prior to migration for the dual purposes of long-duration flight and subsequent egg production. Such a strategy may be particularly important for long-distance migrants that must lay their clutch soon after they arrive on breeding grounds.

Could birds compensate for lower antioxidant concentration?

Although we found that diet supplementation with antioxidants and exercise decreased deposition of lipid-soluble antioxidants to eggs, we did find another surprising effect: supplemented exercised birds invested the most mass in later-laid eggs (Fig. 3C). In the non-supplemented group, regardless of exercise treatment, eggs had the same mass regardless of when they were laid. In contrast, egg mass increased with egg order for supplemented birds, and a post-hoc test showed that the increase was even more

pronounced in the exercised supplemented group. For 5 of the 9 clutches in the supplemented group and 1 clutch in the non-supplemented group, the last-laid egg contained equal or greater total values of lutein and/or vitamin E than the first-laid egg, suggesting that some females were able to compensate for lower yolk concentrations of lipid-soluble antioxidants by increasing total mass of yolk. It is also likely that the heavier later-laid eggs across all clutches in the supplemented group contained more energy or other nutrients that we did not measure than first-laid eggs, and therefore other compensatory mechanisms were at work. This differential allocation across the clutch could impact offspring fitness by improving the survival of later-hatched nestlings, which are typically disadvantaged in this asynchronously hatching species (Royle et al., 2003; Rutstein et al., 2004; Williamson et al., 2006, 2008). We did not allow any eggs to hatch in our experiment, and so the effects of parental exercise and polyphenol supplementation on offspring survival, growth, and fitness remain an open question.

Conclusion

Our experiment provides several key insights into the physiology of volant, breeding songbirds. First, we explicitly demonstrated that exercised birds had higher oxidative damage than non-exercised birds after 6 wk of flight training. Second, consumption of water-soluble flavonoids, antioxidant compounds commonly found in wild foods, decreased the deposition of lipid-soluble antioxidants (i.e., lutein and vitamin E) to eggs, and decreased relative yolk size. For the first time, we provide evidence that

simultaneous consumption of water- and lipid-soluble antioxidants inhibits absorption and thus allocation of lipid-soluble antioxidants to songbird eggs. Third, we show for the first time that flight exercise by songbirds had a carry-over effect on reproduction, lowering deposition of lutein to eggs. These findings have important implications for future studies of wild birds during migration and other oxidative challenges.

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Competing interests

No competing interests declared.

Author contributions

MMS and SRM designed the experiment and edited the manuscript; MMS performed the experiment, conducted the plasma and liver assays, analyzed the data, and prepared the manuscript. NPS, TY, and HM conducted the egg assays and edited the manuscript.

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Data availability

We will archive data in the Dryad repository after manuscript acceptance.

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Table 4.1. Type III tests of fixed effects for plasma oxidative damage (as measured by the d-ROMs test); *P* values ≤ 0.05 are bold.

Effect	Num DF	Den DF	*F* Value	*P* value
Diet	1	25	0.08	0.7807
Exercise	1	25	3.27	0.0826
Sex	1	25	4.14	0.0525
Time	2	50	0.06	0.9457
Diet × Exercise	1	25	1.36	0.2554
Time × Diet	2	50	1.40	0.2570
Time × Exercise	2	50	0.70	0.5021
Time × Sex	2	50	0.51	0.6056
Time × Diet × Exercise	2	50	1.11	0.3382

Table 4.2. Type III tests of fixed effects for plasma antioxidant capacity (as measured by the OXY-Adsorbent test); *P* values ≤ 0.05 are bold.

Effect	Num DF	Den DF	*F* value	*P* value
Diet	1	32	1.50	0.2293
Exercise	1	32	0.11	0.7470
Sex	1	32	0.86	0.3600
Time	2	64	3.87	**0.0260**
Diet × Exercise	1	32	1.27	0.2680
Time × Diet	2	64	1.79	0.1751
Time × Exercise	2	64	2.84	0.0660
Time × Sex	2	64	0.54	0.5857
Time × Diet × Exercise	2	64	2.34	0.1050

Table 4.3. Type III tests of fixed effects for a mixed GLM predicting concentration of lutein in yolk (μg g^{-1}); *P* values ≤ 0.05 are bold.

Effect	Num DF	Den DF	*F* Value	*P* value
Order	1	61.7	47.67	**<.0001**
Diet	1	40.1	7.91	**0.0076**
Exercise	1	40.1	1.71	0.1990
Diet × Exercise	1	40.1	11.07	**0.0019**
Order × Diet	1	61.7	5.27	**0.0251**
Order × Exercise	1	61.7	1.82	0.1824
Order × Diet × Exercise	1	61.7	7.82	**0.0069**

Table 4.4. Type III tests of fixed effects for a mixed GLM predicting concentration of vitamin E in yolk (μg g^{-1}); *P* values ≤ 0.05 are bold.

Effect	Num DF	Den DF	*F* Value	*P* value
Order	1	61.9	39.34	**<.0001**
Diet	1	37.2	11.57	**0.0016**
Exercise	1	37.2	0.06	0.8156
Diet × Exercise	1	37.2	0.40	0.5309
Order × Diet	1	61.9	10.79	**0.0017**
Order × Exercise	1	61.9	0.00	0.9606
Order × Diet × Exercise	1	61.9	0.31	0.5803

Table 4.5. Type III tests of fixed effects for a mixed GLM predicting egg mass; *P* values ≤ 0.05 are bold.

Effect	Num DF	Den DF	*F* value	*P* value
Order	1	18.7	15.66	**0.0009**
Diet	1	25.4	_6.10	**0.0205**
Exercise	1	25.4	_0.17	0.6836
Diet × Exercise	1	25.4	_0.28	0.6009
Order × Diet	1	18.7	12.77	**0.0021**
Order × Exercise	1	18.7	_3.95	0.0617
Order × Diet × Exercise	1	18.7	_2.83	0.1094

Table 4.6. Least square means ± s.e.m. of proportion of yolk (% of whole egg mass) in eggs laid by females that were fed diets supplemented or not supplemented with dietary antioxidants, and that were flown for 2 h each day for 6 wk or were not flown.

	Supplemented	
Exercised	Yes	No
Yes	26.31 ± 0.99	28.57 ± 0.83
No	26.94 ± 0.83	30.27 ± 0.83

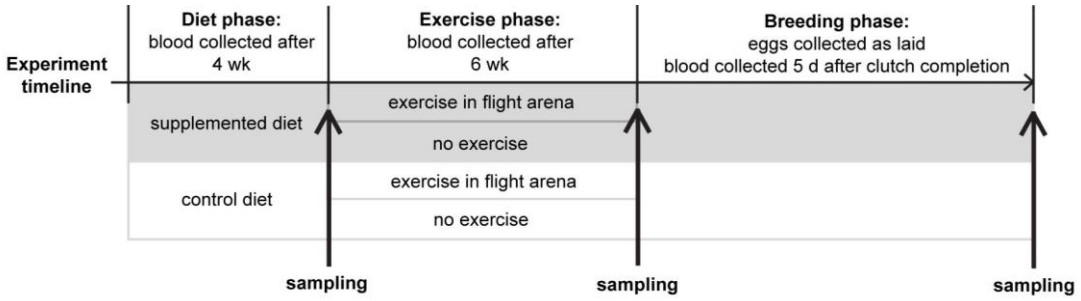

Figure 4.1. **The experiment consisted of three phases: a diet phase, exercise phase, and breeding phase.** We collected blood from the same individuals three times during the experiment and eggs as they were laid; liver tissue was collected from additional individuals after the exercise phase.

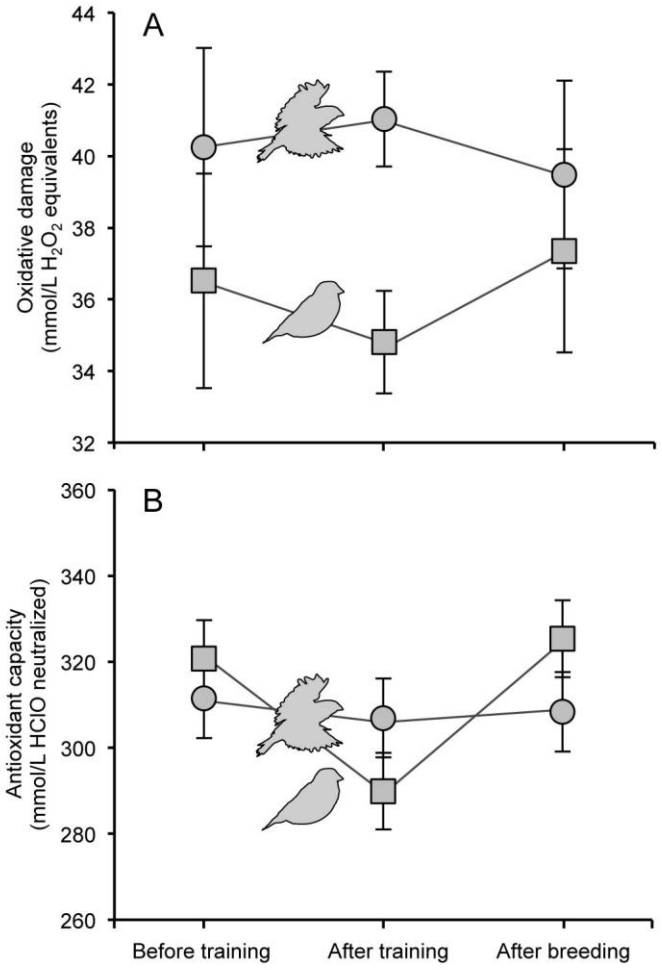

Figure 4.2. **Plasma oxidative damage (A; as measured by the d-ROMs test) and plasma antioxidant capacity (B; as measured by the OXY-Adsorbent test) by exercise treatment, in adult zebra finches during three sequential experimental phases.** Birds were either subjected to 6 wk of exercise training (n = 16 plasma samples for damage, n = 18 for antioxidant capacity; circles) or not exercise trained (n = 14 for damage, n = 19 for antioxidant capacity; squares). Exercised birds displayed higher damage levels than non-exercised birds after training (repeated measures ANOVA, test of effect slices, $P = 0.0025$). Values are shown as least square means ± s.e.m.

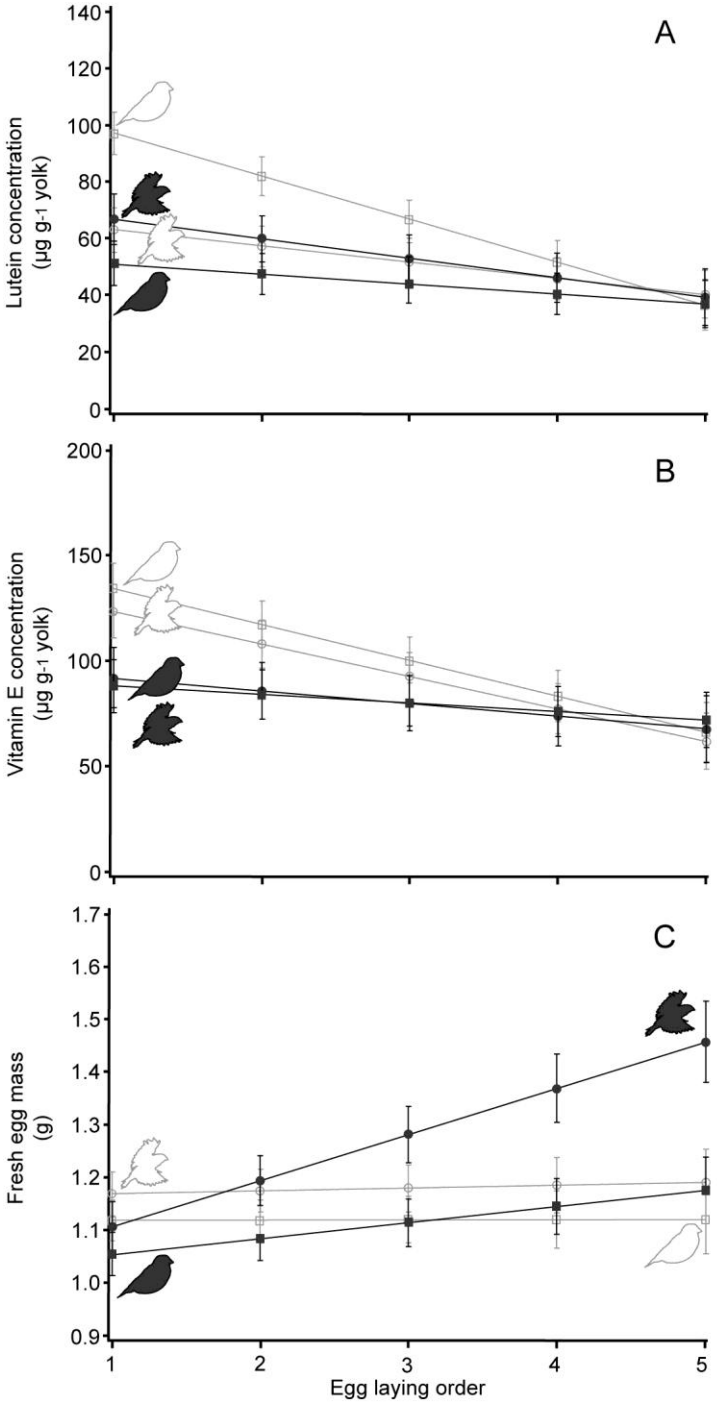

Figure 4.3. **Yolk lutein concentration, yolk vitamin E concentration, and fresh egg mass varied by treatment group in adult zebra finches.** Zebra finches were randomly paired within treatment groups, and the first 5 eggs of each pair's clutch were analyzed. (A) Lutein concentration (µg g^{-1} yolk) decreased with laying order with a more negative slope in the non-supplemented non-exercised group relative to the other groups (mixed general linear model, $P < 0.05$). (B) Vitamin E concentration (µg g^{-1} yolk) decreased with laying order with a more negative slope among non-

supplemented pairs relative to supplemented pairs (mixed general linear model, $P < 0.05$). (C) Egg mass (g) increased with laying order for supplemented pairs, with a more positive slope among supplemented pairs that were also exercised (mixed general linear model, $P < 0.05$). Open squares (open sitting bird) = non-supplemented non-exercised, n = 6 clutches; closed squares (closed sitting bird) = supplemented non-exercised, n = 5 clutches; open circles (open flying bird) = non-supplemented exercised, n = 5 clutches; closed circles (closed flying bird) = supplemented exercised, n = 4 clutches. Values are shown as least square means ± s.e.m.

CHAPTER 5

Crafting and evaluating Broader Impact activities:

a theory-based guide for scientists

by

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Abstract

To secure research funding from grant-awarding agencies such as the US National Science Foundation, scientists – despite not typically being trained in non-technical communication or public engagement – must competitively formulate so-called Broader Impacts activities. Dissemination activities are often proposed as Broader Impacts of research, but what characteristics of these activities truly indicate their potential to be "broad" or "impactful"? How can the "impacts" of very different activities be fairly compared during peer review? Combining the experiences of successful practitioners with communication theory, I have synthesized a five-point framework that could help both proposers and reviewers craft and compare Broader Impacts dissemination activities. This "Broader Impacts Impact Framework" summarizes best practices in communication and outreach, and can be easily used by scientists during proposal writing and review. This framework focuses on five main factors: who, why, what, how, and with whom.

In a nutshell:

- Scientists often struggle to formulate effective Broader Impacts activities for their funding proposals
- Communication theory may offer helpful strategies
- All successful dissemination activities share common characteristics
- Highlighting just five of these characteristics in proposal descriptions could help peer reviewers to judge and compare the potential for impact among a range of proposed Broader Impacts activities

Scientists and those who evaluate proposed research requiring funding (hereafter "reviewers") have expressed the view that – during the US National Science Foundation (NSF) proposal process – fulfilling the Broader Impacts (BI) criterion is more challenging than addressing the Intellectual Merit (IM) criterion (NSB 2011). True, scientists' expertise generally lies predominantly in the subject matter addressed by IM, but perhaps the greatest problem is that, in contrast to the IM criterion, no standardized framework exists for evaluating BI activities. BI spans a wide spectrum of potential outcomes (top of Figure 5.1), with "dissemination of research findings to increase scientific literacy" being the third most proposed category across all NSF directorates (after "teaching/training" and "broadening participation of underrepresented groups"; NSB 2011). How can these very different dissemination efforts be fairly compared?

Reviewers look for particular characteristics in the IM section of a proposal, regardless of the subject of the research project, and all competitive IM descriptions display certain qualities: for instance, appropriate and rigorous research design with suitable sample size and controls, a solid theoretical foundation for the work, the potential to substantially advance understanding, and evidence that the proposer is knowledgeable and has the resources to carry out the study. The latter two characteristics are even listed in the NSF Proposal and Award Policies and Procedures Guide (NSF 2013). Essentially, principal investigators (PIs) know that the IM sections of their proposals must demonstrate particular qualities, and reviewers are aware that they must look for them.

Yet what should PIs demonstrate or emphasize in their BI descriptions to convince the reviewer that their proposed activity will be truly "broad" or "impactful"? Guidance from NSF remains sparse regarding best practices in the crafting and judging of BI activities (NSF 2007, 2013). A common goal of many BI activities, however, is skill-building or wide dissemination of knowledge, with the intention that research-generated information or new skills will be used outside of the original research group; for convenience, I will hereafter call these varied efforts "outreach", because they typically extend beyond the PI's research program (bottom of Figure 5.1). My goal has been to answer the following question: what qualities characterize broad and impactful outreach activities, making them more effective in practice and potentially more competitive in peer review? Although I focus on NSF and the BI criterion, the principles I describe here can be applied to any effort intended to disseminate or use scientific research outside the research group where it was generated. By examining examples of successful information-dissemination programs, cautionary tales, and theoretical work, I have created a standardized framework of characteristics that may help proposers and reviewers craft and compare BI activities that focus on bringing research-generated information to various audiences. I call this a Broader Impacts Impact Framework (formerly "Factor"; Skrip in press), or BIIF for short.

The BIIF

The BIIF (Table 5.1) consists of five straightforward categories of characteristics that scientists should carefully consider when evaluating the potential for success of their

BI outreach activities. For example, supposing someone in my research subdiscipline – songbird migration, nutrition, and physiology – proposes BI outreach activities that will (1) engage citizen scientists in data collection, (2) bring their science to public lectures or workshops, or (3) detail the results of their work online or in non-scientific publications (Figure 5.2). Imagine that a reviewer encounters three competing proposals, with equally competitive IM, each of which suggests one of these types of activities. How might that reviewer judge which among them is best, or determine whether any of the activities are likely to be "impactful"? The BIIF synthesizes theory and best practices to help answer these questions by calling attention to the following straightforward considerations: who, why, what, how, and with whom.

Who is the audience?

Descriptions of BI activities in proposals should define a target audience and be as specific as possible. When it comes to potential for "impact", all audiences are not equal. Proposers should be prepared to defend their choice of audience and state why that audience is the most important one to reach because they are the most likely to use and spread the science-generated information.

Evidence suggests that, to have the greatest effect regardless of activity type, PIs should focus their outreach efforts on so-called "gatekeepers" or "opinion leaders", individuals or institutions that exert the most influence and therefore have the greatest potential to affect policies and practices, spread a scientific message, or serve as a trusted conduit for some call to action (Heberlein 2012; Clayton *et al*. 2013). Gatekeeping/opinion-leading audiences – including policy makers, community

program administrators, educators at informal learning centers, religious leaders, teachers, and wildlife managers – are perhaps in the best position to make use of information that is disseminated by scientists, and can serve as trusted, familiar conduits for a message (Khalil and Ardoin 2011; Purcell *et al*. 2012; Trautmann *et al*. 2012; Jordan *et al*. 2013). After all, how people receive and use information depends on how much they trust the source, and personal communication among individuals in social networks remains a primary vehicle for messages and for recruiting people into activities or ways of thinking (Besley *et al*. 2008; Cronje *et al*. 2011; Chu *et al*. 2012).

How could this idea be used during proposal preparation and peer review? Returning to the hypothetical example of the three competing BI activities (Table 5.2a), we must first keep in mind that the "best" audience for a dissemination activity depends on what that activity is trying to accomplish. So if, for example, the proposers' aim is to promote conservation of bird habitats and the planting of fruiting shrubs that many songbirds use during migration, in the context of a BIIF, a very strong BI section will identify a specific audience of gatekeepers or opinion leaders to whom the dissemination activity will be initially directed, while a weak BI section will not (Table 5.2a).

Why propose a particular activity?

The widespread notion among scientists that simply providing scientific information will change public opinions or help the public solve environmental problems remains a fallacy that undermines the very mission of the BI criterion. Communication professionals and social psychologists have long understood that the "deficit model"

approach to science outreach (wherein the public is seen to have an "information deficit", fixable by provision of data) is ineffective in accomplishing educational goals or achieving lasting attitudinal/behavioral changes (Gross 1994; Besley and Tanner 2011; Heberlein 2012). Yet scientists have been slow to espouse this view, mainly because they are not trained to consider different models of knowledge-building, the values and roles of non-scientific expertise, and the competing factors and filters that affect how scientific information is assimilated and used by non-scientists. The "public" is not an empty vessel waiting for scientific knowledge; rather, the varied and complex social needs of different audiences must drive the outreach efforts of scientists.

Returning to the hypothetical example (Table 5.2b), if a PI's proposal claims that s/he intends to "educate the public" through BI activities, reviewers should be wary. To what end is the PI trying to "educate the public"? Who is "the public"? Is this "education" meant to bring about a behavioral change through one-way provision of scientific information? If so the chances are high that it will not work. Pro-environmental change does not depend on ecological understanding alone; it also depends on the non-ecological values (aesthetic, economic, etc) that an audience holds, irrespective of their comprehension of the science (Hager *et al*. 2013). Rather than focusing on "knowledge gaps", BI activities should propose solutions and actions in which audience members can be engaged, to improve their own lives and environment, in accordance with their existing values. Aims that are highly specific are more likely to come to fruition (eg Roberts 2009) and should be more highly valued in proposal ranking.

If a proposer intends to increase knowledge, without any unrealistic expectation that a behavior change will follow, s/he should be able to identify specific knowledge gains that are planned. Defining and measuring changes in scientific literacy can be problematic, especially if the audience is already relatively scientifically literate; thus, if a proposer seeks to increase science (including ecological) literacy, reviewers should be suspicious unless literacy in a particular topic area is specified (Cronje *et al*. 2011; Phillips *et al*. 2012). Essentially, means of knowledge dissemination should be appropriate for the type of knowledge they are meant to improve and suited to the "why" behind the effort (Table 5.2b).

What should a BI activity involve?

Ideally, proposers should be able to demonstrate that they will consider the needs of their audience. Several "key themes" emerge from the literature: (1) promoting self-empowerment, (2) exchange of ideas, (3) value of non-scientist opinions, (4) interactivity, (5) personal contact, and (6) performing a service (see below). Essentially, when hoping to successfully deliver a message to a specific audience, scientists must strive to understand that audience and what its members want.

When presenting the public with a description of an environmental problem, self-empowerment and agency – the notion that an audience member can do something relevant and that her/his actions will matter – should be stressed (key theme 1) (Koepfler *et al*. 2010; Jordan *et al*. 2012a; Clayton *et al*. 2013). Additionally, the recipients of outreach efforts must have the opportunity to contribute feedback at all stages of project development (key themes 2, 3, and 4) (Dickinson and Bonney 2012;

Druschke and Seltzer 2012). To promote retention and audience satisfaction, for example, the Cornell Lab of Ornithology's citizen-science programs treat their participants as customers, and attempt to provide the best "service" to keep those participants engaged (key themes 5 and 6) (Chu *et al*. 2012; Trautmann *et al*. 2012). To have the greatest effect, BI activities should strive to do the same, by identifying the unique needs, attitudes, and motivations of their audience and by aiming to supplement that audience's knowledge and skillset with research-generated information and skills (Gross 1994). Of course, this requires that PIs recognize how much their audience already knows and can do (Petts and Brooks 2006). To my knowledge, no how-to document currently exists that advises scientists on how best to complement their expertise with target audience expertise (eg how to best mesh the knowledge base and skillsets of researchers and non-scientists). However, Frechtling (2010) offered strategies for identifying audience attitudes before projects begin and for evaluating project outcomes. Ideally, any proposal would reflect a clear understanding of the audience's motivations, as well as plans to modify the suggested approach based on audience feedback.

Any of the three BI activities that were previously introduced in the hypothetical bird nutrition example – citizen-science outreach, public workshops and lectures, or non-scientific publications – can display the key themes discussed earlier if their proposers are sufficiently creative (Table 5.2c). According to the BIIF, a very strong proposal description would indicate that the BI project will seek feedback from the audience, promote personal two-way communication, and identify what the audience can do about a particular science-based problem.

How does an activity truly affect an audience?

BI activities with the highest potential for impact are those that accommodate human nature. Scientists may be tempted to claim that an audience "should" do, know, think, or value a certain "something" related to their work; but rather than tell people what they should care about, truly effective programs describe how science is relevant to what people already value, believe, or do (Bonter 2012; Chu *et al*. 2012; Heberlein 2012; Purcell *et al*. 2012). Outreach efforts that adopt this approach tend to follow the three guiding principles discussed by social psychologist Thomas Heberlein (Heberlein 2012) in the context of pro-environmental campaigns: the "direct experience principle", the "identity principle", and the "specificity principle". They could easily – and should – be integrated into the way proposals are prepared and compared.

First, an audience is more likely to learn a particular skill or adopt a particular attitude or behavior if they have had direct experience with the phenomenon in question and can relate to it on a personal level (Bonney and Dickinson 2012; Heberlein 2012; Jordan *et al*. 2012b; Oberhauser 2012; Reynolds and Lowman 2013). As much as possible, outreach efforts must therefore provide direct experience (eg, encourage or facilitate data collection or other observations, solicit artwork or other participatory creative works, or offer opportunities to contribute to a specific outcome), or remind the audience of a relevant direct experience they themselves have had (eg, visiting, making, or observing something in their own lives).

Second, the most ingrained attitudes and behaviors are those most closely tied to an individual's own sense of personal identity or ownership; deeply emotional values

can reframe how an individual interprets a science-based message (Laslo *et al*. 2011; Heberlein 2012). Consequently, an audience's established worldview must be taken into account before a scientist begins an outreach effort, as that worldview is unlikely to change in the face of new information. Fortunately, identity-based attitudes can be powerful allies when efforts are crafted to draw on the pride that audiences take in themselves and their communities (eg Purcell *et al*. 2012; Hager *et al*. 2013).

Finally, the specificity principle suggests that audiences do not necessarily behave in ways consistent with their own attitudes, given the myriad factors that determine their day-to-day actions and decisions. If a scientist wants to increase the popularity of a particular pro-environment or pro-science behavior (eg using energy-saving technologies or reporting bird sightings) among a particular audience, it is important to target and facilitate that behavior. Facts alone do not solve problems; people act when provided with a sense of self-empowerment and agency (as mentioned above) and a sense of free choice, which helps to prevent them from resenting an expert's directions or advice (Heberlein 2012).

So, according to the BIIF (Table 5.2d), a very strong BI section will describe activities that provide an audience with a direct experience, induce them to recall one, or give them the sense of a personal stake in the science. Ideally, it also will appeal to the audience's sense of place or ownership, will focus on a particular behavior, and will provide or suggest the means by which the audience can achieve it.

With whom is the activity to be designed or performed?

Scientists need not be skilled in outreach to have an impact; indeed, it is arguably an

extra burden on them to obtain those skills. Instead, partnering with social scientists (including social psychologists), professional communicators, artists, filmmakers, museum staff, and/or educators can help to promote synergy among different professionals already equipped with the necessary skills to design impactful outreach efforts. All of these individuals are experts in message formulation and delivery and can be valuable resources and collaborators. Scientists should avoid "reinventing the wheel" by working with pre-existing infrastructure – ie pre-established staff, community groups, institutions, or partnerships – to achieve dissemination goals.

This idea of partnership is not new, but it should be stressed during proposal preparation and review. As Burggren (2009) pointed out, if the ultimate goal of the BI criterion is to pair effective outreach with high-quality science, rather than transform the abilities or philosophies of scientists, individuals who are trained in outreach and public education should be the ones actually doing the outreach and education. For instance, museums and other educational institutions provide pre-existing infrastructure for grant-funded scientists, so as to satisfy the BI criterion and also to maximize the acceptance of their message, given that these institutions are typically considered as trusted and politically neutral venues for free-choice learning (Alpert 2009; Khalil and Ardoin 2011). Intra-institutional bodies can also offer professional outlets for academics to gain the most impact from their BI activities; boundary organizations such as extension services, university communications offices, and supporting resources for faculty within academic institutions specialize in the outreach skills that PIs may lack or simply do not have time to exercise (Roberts 2009; Dickinson and Bonney 2012).

Furthermore, when social scientists can study the success or progress of BI activities as they are carried out, changes to the activities' format or approach can be made mid-project and future activities can be improved based on their findings (Burggren 2009; Frodeman and Parker 2009; Druschke and Seltzer 2012). Many methods and criteria exist for evaluating these activities, and should be chosen to suit the project at hand (Rowe and Frewer 2004; Frechtling 2010). Such an iterative evaluation approach parallels the "adaptive management" strategies already familiar to ecologists; much can be gained from studying how a project is working while it is still ongoing.

According to the BIIF (Table 5.2e), therefore, a very strong proposal description will demonstrate that the PI either has proven experience in successful outreach project design/execution or has partnered with an individual or group who does. In particular, collaborations with social scientists who will study the BI activity, and with institutions or offices that already have strong relationships with non-scientist communities, should be of great value.

Conclusion

At the heart of the BIIF is the idea that, regardless of the BI outreach activity, a strong proposal will display certain elements that indicate a high potential for impact. If the five categories of qualities described above (Table 5.1) are addressed in a funding proposal, scientists and reviewers can compare a wide range of activities. This is not intended to promote certain BI dissemination activities over others, as long as they accomplish the desired outcome – that is, the impact they are meant to have.

BI activities should aim to:

- target a specific audience that can make practical use of the proffered, research-generated information and include the potential for ongoing effects through non-target audiences (eg if the target audiences are educators, policy makers, or wildlife managers);
- achieve an outcome with a contextual rather than an "information deficit" approach;
- communicate self-empowerment and encourage personal contact and feedback;
- accommodate human nature by considering Heberlein's (2012) direct experience, identity, and specificity principles; and
- integrate with existing outreach programs that offer a diverse range of additional skills.

Any dissemination activity – ranging from citizen data collection, to public lectures and workshops, to popular online and print publications, and more – could be effective if their proposers explicitly addressed these points. In this way, disparate BI activities can be compared, not by making value judgments about the different media and techniques they use or by tallying audience numbers, but by carefully examining whether they fulfill the basic criteria that communication professionals and social psychologists have described in their varied discussions of successful impact.

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Table 5.1. The Broader Impacts Impact Framework (BIIF)

(1) **Who** is the audience for the activity?
How was the audience chosen?
Does the audience include "gatekeepers" and/or "opinion leaders"?

(2) **Why** was this particular activity chosen?
Does the activity perpetuate the myth of information deficit
(ie that information is enough to promote behavior or policy changes)?
Does the proposal specify a particular objective to be met?

(3) **What** does the activity involve?
Does the activity incorporate the following:
Audience self-empowerment?
Exchange of ideas/interactivity/personal contact?
Value of non-scientist opinions/contributions?
Serving a public need?

(4) **How** will the activity accommodate human nature?
Does the activity incorporate the following:
Direct experience?
Audience's sense of identity?
Specificity of action?

(5) **With whom** is the activity to be designed or performed?
Does the proposal demonstrate prior experience in successful outreach?
Does the activity involve collaboration with social scientists, professional communicators, or other intra- or extra-institutional staff?

Notes: The BIIF consists of five categories of qualities that characterize "impactful" outreach activities; this framework can help proposers to craft, and reviewers to compare and rate, Broader Impacts outreach activities.

Table 5.2. Three hypothetical competing proposals with different proposed Broader Impacts outreach activities

	High potential for "impact"	Low potential for "impact"
(a) **Who**	Proposal identifies specific audience that can further spread the message (eg "Our audience includes leaders and members of specific bird or garden clubs, such as…").	No description is given of the best conduit audience for the activity (ie gatekeepers or opinion leaders who could more likely influence others).
(b) **Why**	Proposal demonstrates that the PI is well acquainted with the needs and attitudes of the audience, and the scope of the activity is realistic (eg "We will draw on the pro-wildlife values of local landowners to encourage pro-bird gardening habits..."). The PI does not expect to bring about behavioral change in a wide, heterogeneous audience by provisioning general science facts.	Proposal displays vague, information-deficit thinking; eg "We will educate the public…", "We will increase general scientific literacy…", or "We will raise awareness…". Educational goals are not specific.
(c) **What**	Proposal indicates that the project will seek feedback from the audience, promote personal two-way communication, and identify "what you can do".	The project's communication will be solely one-way, with no knowledge-building among the audience or promotion of self-empowerment.
(d) **How**	The project will provide direct experience, appeal to a sense of "ownership" or "place" (eg "my" garden, "my" town, "my" backyard birds), and/or identify the means to achieve a specific behavior (eg "We will highlight where landowners can buy the kinds of shrubs that birds use during migration, to plant at home...").	The proposal makes no mention of direct experience, consideration of audience identity, or specific educational or behavioral targets.

(e) **With whom**	The PI has an intra-institutional (eg communication department) or extra-institutional (eg museum, school) partner. A social scientist will study the activity's outcomes.	A PI without communication expertise makes no effort to collaborate with a communication specialist.

Notes: In this hypothetical example, a reviewer considers three proposals from different principal investigators (PIs) with equally competitive Intellectual Merit, but proposing three different Broader Impacts activities (citizen participation in data collection, lectures/workshops, and online or print articles); use of the Broader Impacts Impact Framework helps distinguish competitive activities with high potential for "impact" from less competitive activities, regardless of the form that activity takes.

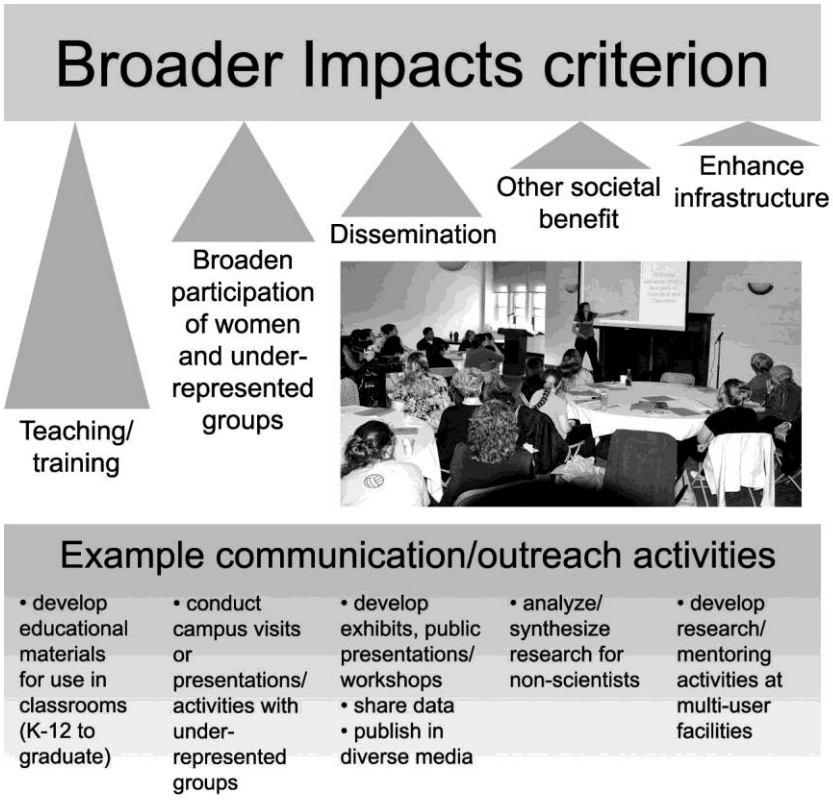

Figure 5.1. The NSF Broader Impacts criterion spans a range of activities and outcomes, typically arranged in five categories. (top) The length of each triangle corresponds to the relative popularity of each category among NSF proposals (NSB 2011). (bottom) Despite the categories' apparent differences, NSF (2007) provided examples, paraphrased here, for each category that relate to the guidance offered in this paper. Inset photograph: the author explains an ecological concept at a local conference.

Inset photo credit: R Morgan

Figure 5.2. In a hypothetical example, three scientists studying the ecophysiology of songbirds propose different Broader Impacts activities in their funding applications: (1) engage citizen scientists in data collection, (2) bring their science to public lectures or workshops, or (3) detail the results of their work online or in non-scientific publications. The Broader Impacts Impact Framework helps distinguish the strengths and weaknesses of such proposed activities, regardless of their different forms.